SYNTHESIS OF HIGHLY QUENCHING FULLERENE DERIVATIVES FOR BIOSENSOR APPLICATIONS

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To my family for all their
unconditional love and support

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ABSTRACT

This dissertation examines the synthesis of fullerene-based fluorescence quenchers for numerous biosensor applications. The Introduction describes the need for biosensors in our society, what they are and various biosensing schemes that are currently being worked on in our group. Chapter One describes the synthesis of a number of fullerene derivatives. In order to incorporate fullerene derivatives into biosensors, they need to posses a functional group that can be easily reacted with biomolecules. Two of the functional groups by which molecules are conjugated to biomolecules such as amino acids and proteins are amines and carboxylic acids. For this reason, we synthesized amine- and carboxy-containing C_{60} that could then be conjugated to biomolecules.

Chapter Two describes the steps taken towards the incorporation of these fullerene derivatives into biosensors. First, Stern-Volmer experiments were conducted to determine whether or not the fullerene derivatives would be good quenchers for our polymers. Second, a polymer with pendant fullerenes was made to determine whether or not there was an enhancement in the quenching as compared with the Stern-Volmer data. Third, the use of the biotin-streptavidin system to determine how well the fullerene derivatives would perform in a biosensor system is discussed.

Thesis Supervisor: Timothy M. Swager
Title: Professor of Chemistry

Table of Contents

There are various pressing problems in today's modern world. Many countries find themselves facing serious terrorist threats. They also find themselves facing the development and spreading of diseases. In a society that is extremely fast-paced and in which time is given a monetary value, solving these problems is winning just half of the battle. Not only the problem must be solved, but it also must be solved quickly. Therefore, effective and fast-working technologies must be developed for the detection of disease-causing agents and explosives, among others. Various promising biosensor devices to target these issues are being developed and optimized by various research groups and private companies. Recently, our group has started working on the development of fluorescence-based polymer biosensors for the detection of cancer, DNA, enzymes and antigens. Several of the biosensor components have been targeted for improvements and this thesis discusses the improvement of the fluorescence quenchers used in our biosensors.

What are biosensors?

In general terms, a sensor is a device that is able to detect a certain substance and produce a signal that can be measured. More specifically, a sensor must be able to distinguish between the target analyte and a vast number of inert and interfering species.[1] A sensor is composed of two main parts that allow for its functioning, a recognition site and a transducer. The recognition site responds to the presence of the target analyte and the transducer converts this response into a different kind of energy that can be amplified, processed and converted into the desired format.[2] A schematic diagram of a sensor is shown in Figure 1.

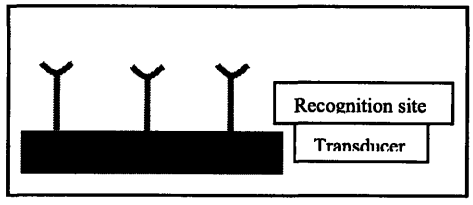

Figure 1. Schematic diagram of a sensor

A sensor must produce at least two different kinds of signals, one when there is interaction with the target analyte and another one when there is none. This is shown in Figure 2. In part a, there is no analyte present and the output of the system is "signal 1". In part b, even though there is analyte present, no interaction is taking place at the moment, so the output is again "signal 1". In part c, there is interaction between the analyte and the recognition site. Therefore, the output is different than in the previous cases ("signal 2").

For some applications, a sensor must be able to recognize the target analyte when its concentration is very low and there are many interfering species present in the sample. For example, the concentration of some proteins in blood serum is around 1 _g/L, while the total protein concentration is 70 g/L.[3] Thus, the sensor should be able to discriminate 1 in $10^7 - 10^8$ in order to specifically recognize the target analyte. This means that the sensor must show a remarkable degree of specificity for the analyte and still retain the appropriate sensitivity to monitor the target analyte in the concentration range at which is found in the sample.[2] This combination of specificity and sensitivity are usually only displayed by biological molecules. When a biological component is utilized in the recognition site, the sensor is then called a biosensor. According to Higgins and Lowe:[1,2] "A biosensor may be defined as a device that recognizes an analyte in an appropriate

sample and interprets its concentration as a signal, via a suitable combination of a biological recognition system and a transducer".

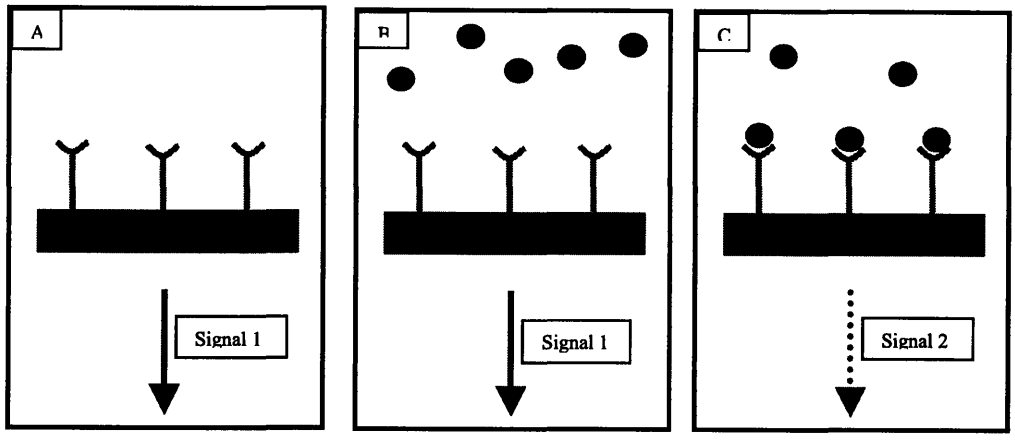

Figure 2. Output of a sensor under different conditions. (a) no analyte present, (b) no interaction with the analyte, (c) interaction with the analyte

The history of biosensors started in 1962[4] and the progenitor of the biosensor was Leland C. Clark.[5] He studied the electrochemistry of oxygen at platinum electrodes, then using platinum electrodes as oxygen sensors. Clark then decided to place glucose oxidase, an enzyme that reacts with oxygen, close to the surface of the platinum electrode. His reasoning was that he could follow the activity of the enzyme by monitoring the changes in the oxygen concentration around it, thus designing the first biosensor.[5] This glucose biosensor has been very well studied and is commercially available for diabetics.[6]

The glucose biosensor shows the application of biosensors to health related issues. The need for analytical information applies to a wide range of activities, not just to health related issues. Some of these areas are food analysis, environmental monitoring and national defense.[7] Here are some specific examples of biosensors in theses areas. Suleiman and Guilbault have developed several biosensors with food analysis applications that include enzyme electrodes and fiber optic probes to detect various

substances such as fructose, glutamate, aspartame, hydrogen peroxide, glucose and sulfite.[8] Sandberg *et. al.* (1992) have developed an enzyme-lniked immunosorbent assay (ELISA) with environmental applications that uses electroconductive polythiophene for the detection of pesticides.[9] Whitten at the Oak Ridge National Laboratory has been developing a biological threat detector using optical spectra with obvious applications on national defense.

Components of a biosensor: a closer look

The biological recognition system recognizes the target analyte and responds with a change in one or more physicochemical parameters associated with the interaction. There are many biological components that can be used at the recognition site of a biosensor, such as enzymes, antibodies, organelles, microorganisms, tissues and cells.[10] Most current biosensors use enzymes or antibodies at their recognition sites.[10] Enzymes are extremely specific at catalyzing reactions: any given enzyme will always turn A into B and never into C.[3] Antibodies are also very specific and respond to the entry of "foreign" material into the body. They do not necessarily catalyze chemical transformations like enzymes, but instead they undergo a physical transformation that can be detected.[3] The main problem with designing the recognition site of a sensor is that the integration of biological components and synthetic elements involves time and labor-consuming chemistry.[11]

The transducer responds to the products of the biocatalytic or binding process that occurs in the recognition site. There are four main types of transducers: potentiometric, amperometric, optical and other devices (Table 1).[2] Potentiometric devices measure the

accumulation of charge density at the electrode surface and work under equilibrium conditions.[10] They have been mostly developed around pH sensitive electrodes and they are applicable to any enzymatic pathway in which the concentration of H^+ changes. Amperometric devices measure faradaic currents that result from the electron transfer between a biological system and an electrode held at an appropriate potential.[8] Optical devices measure the interaction of light with the sample. Other devices such as thermistors, surface conductance probes and piezoelectric or surface acoustic wave devices can measure enthalpy, ionic conductance and mass.[2]

Table 1. Classification of established transducers[2]

Class	Examples
Potentiometric	Ion-selective electrode, ion-selective field effect transistor, gas-selective electrode
Amperometric	Metal electrodes, mediated systems, condicting organic salts
Optical	Absorption, fluorescence, ellipsometry, planar waveguide, fiber optic, surface plasmon resonance
Other	Thermistor, surface conductance, piezoelectric/surface acoustic wave

The most sensitive optical sensors are based on the use of fluorescence as the transduction method.[8] A recognition event that produces a diminution, improvement or a shift in the emission wavelength can be used for the production of a functional sensor.[12] Some of the advantages of using fluorescence as the transduction method are that it is a property that is easy to measure and that the measurements can be done fast.

Many different polymers have been synthesized in our group and it has been shown that these fluorescent polymers can enhance the sensitivity of sensors.[13] The reasoning is that having a polymer is like having many sensory subunits liked together.

Our group has shown that this "molecular wire" approach produces signal amplification when compared to single molecule systems.[13,14] A schematic diagram of the molecular wire approach is shown in Figure 3.

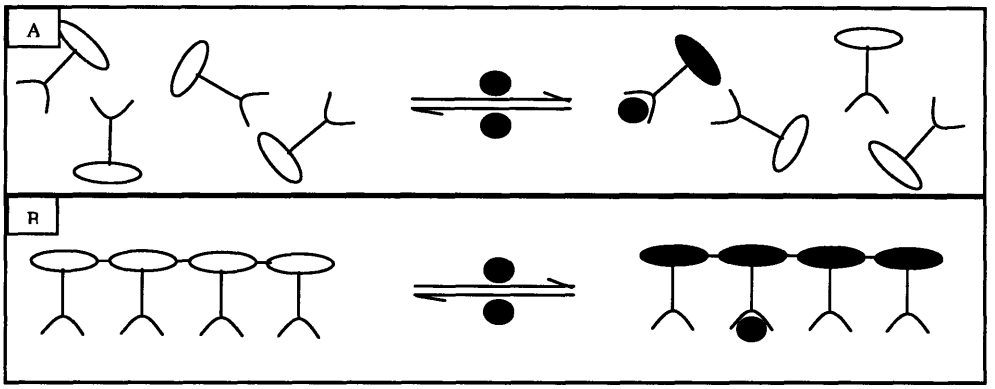

Figure 3. Traditional sensor (a) and the molecular wire approach (b).[10]

Polymer-based sensors

There are two main types of polymer-based sensors, turn-off and turn-on sensors (Figure 4). In a turn-off sensor, a migration of excitons through the polymer backbone is quenched when electron transfer to a suitable acceptor occurs.[15] This results in amplified quenching. In a turn-on sensor, a non-quenching analyte causes a local minimum in the bandgap and the recombination of excitons.[15] This results in amplified wavelength shifts.

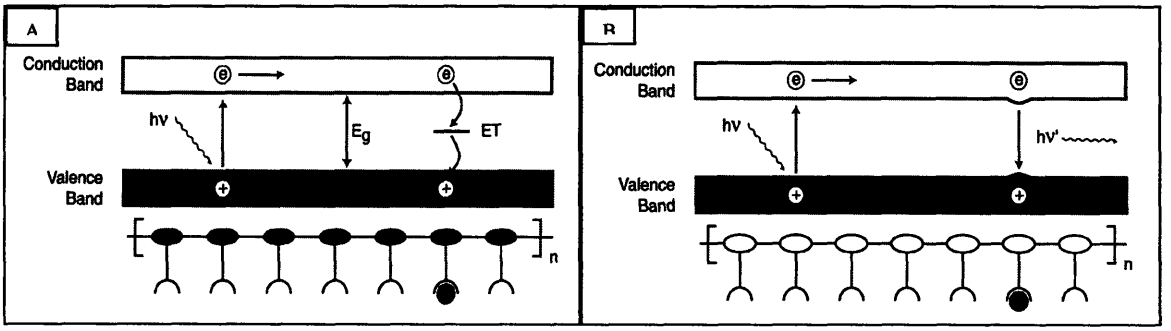

Figure 4. Polymer based turn-on (A) and turn-off (B) sensors.[15]

There are many biosensor applications of conjugated polymers. Three applications that our research group have worked with or is working with are simple energy transfer, the turn on of fluorescence by quencher removal and the detection of co-localization.[16] These applications are shown in Figure 5. In 5a, simple energy transfer is shown. In this case, the polymer has a receptor group that can bind the target analyte, which can be DNA, an antibody, a protein, etc. The conjugated polymer serves as a light-harvesting unit and upon binding, a new emission is obtained from the system. In 5b, the turn-on of fluorescence by quencher removal is shown. In this case, a quencher is attached to the conjugated polymer. After the removal of the quencher by enzymatic hydrolysis, a strong emission from the conjugated polymer is obtained. In 5c, the detection of co-localization is shown. In this case, there is energy transfer between the conjugated polymer and a suitable energy acceptor that is in close proximity. This results in amplified detection of the spatial interactions between biomolecules.

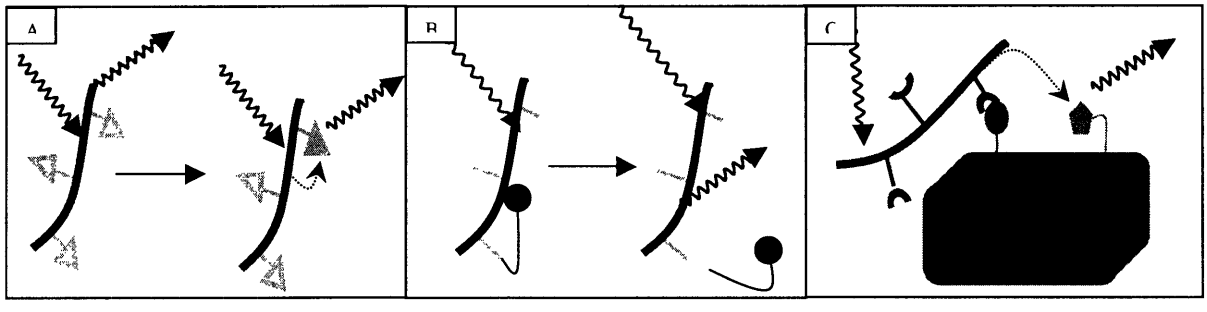

Figure 5. Bisensor applications of conjugated polymers: simple energy transfer (A), turn-on of fluorescence by removal of quencher (B) and detection of co-localization (C).[16]

Fluorescence quenchers

Any process that decreases the fluorescence intensity of a sample is called fluorescence quenching.[17] Some of the molecular interactions that can result in quenching are excited-state reactions, molecular rearrangements, energy transfer, ground-

state complex formation, and colisional quenching. Some examples of quenchers are oxygen, halogens, amines, and electron-deficient molecules.[17]

This thesis will deal with the development of fullerene based fluorescence quenchers for various applications in biosensors. As stated above, quenchers are an essential component of fluorescence turn-on biosensors. Fullerene-based quenchers are chosen because they should exhibit strong electronic interactions with the polymers studied in our group. These strong electronic interactions should result in an enhancement in the quenching. The development of a very effective quencher would result in a general enhancement in the sensitivity of the biosensor. A more detailed explanation of fullerenes and fluorescence quenching is included in the following chapters.

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The discovery of the fullerenes and, more specifically, their availability in macroscopic quantities, created considerable of excitement among the scientific community. The possible applications for these three-dimensional, all carbon molecules were numerous. Unfortunaltely, due to solubility problems, these molecules have not been as useful for applications as researchers first thought. Nonetheless, these solubility problems can be partially solved by derivatization. Various different reactions for the derivatization of the fullerenes have been explored. This introduction attempts to provide a brief summary of the synthetic advances in fullerene production and derivatization since their discovery in 1985 until now.

Historical Background

The discovery of the fullerenes wasn't exactly rocket science, but there is an interesting relationship between their discovery and space. In the early 80's, the study of refractory clusters was revolutionized by the laser vaporization beam technique developed by Rick Smalley.[1] This technique allows the simulation of stellar nucleation conditions if graphite were vaporized.[2] Robert Curl and Rick Smalley collaborated to study cyanopolyynes,[3] using the laser beam vaporization technique. The cyanopolyynes are long carbon chain molecules that stream out of red giant carbon stars.[4] With these experiments, it was discovered that cyanopolyynes are formed in a plasma by a laser focused on a graphite target.[3] C_{60} and its remarkable stability were also discovered![5] The stability of C_{60} was rationalized on the basis of a structure with the symmetry of a soccerball.[5] The molecule was named Buckminsterfullerene after the designer of the geodesic domes[3] because the stability of the C_{60} was due, in part, to geodesic factors.

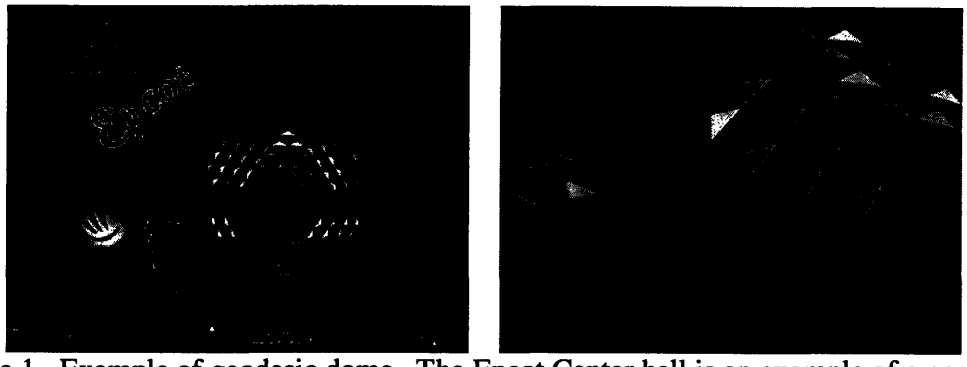

Figure 1. Example of geodesic dome. The Epcot Center ball is an example of a geodesic dome. A geodesic dome is defined as a dome composed of other geometric figures.

It must be mention that there were earlier reports in literature of the C_{60} molecule. The first article about this molecule appeared in 1970 in *Kagaku*[6] and was written by Eiji Osawa. He predicted a molecule like C_{60} would be stable. The following year, Osawa and Yoshida described such a molecule in more detail.[7]

What are fullerenes?

The fullerenes are all-carbon molecules that contain 2(10 + N) carbon atoms, which are divided into 12 five-membered rings and N six-membered rings. This building principle arises as a consequence of the Euler's theorem, which predicts that 12 pentagons are needed for the closure of a carbon network with N hexagons.[8] In theory, at least one fullerene structure can be formed by any even-numbered carbon cluster with more than 20 carbons (except for C_{22}).[8]

The smallest stable fullerene, and also the most abundant, is C_{60} and its stability can be explained by the fact that it is the smallest fullerene to obey the isolated pentagon rule (IPR).[9] The IPR establishes that structures in which the five-membered rings are completely surrounded by six-membered rings are more stable because of strain and electronic arguments. Other fullerenes that obey this rule are C_{70}, C_{78} and C_{84}.[8]

Synthesis of fulllerene C_{60}

Buckminsterfullerne was discovered in 1985, but macroscopic quantities of it, were not available until 1990.[10] There are various ways of producing fullerenes: arc heating of graphite[11], inductive heating of graphite[12], the use of solar generators[13,14], combustion[15] and pyrolysis of naphthalene[16]. Of all these options, the most effective one is the resistive heating of graphite, which was also the first technique used to produce macroscopic quantities of C_{60}.[10] In this technique a voltage is applied between two graphite rods under He where the evaporated carbon atoms cluster and anneal to give C_{60}, among other fullerenes in yields of 5-10%.[17]

C_{60} and other fullerenes are now commercially available from various vendors like Texas Fullerenes Corporation, MER Corporation, SES Research and Hoechst AG. The prices are around $800 for 5 grams of compound.

Solubility of C_{60}

The major obstacle to using C_{60} for different applications is its solubility. C_{60} is insoluble or only sparingly soluble in most organic solvents.[18] The C_{60} also aggregates easily, which makes it even less soluble.[19] To partially overcome this obstacle, the C_{60} can be derivatized. Its chemistry is discussed in the following section.

Table: Solubility of C_{60} in commonly used organic solvents (T = 298 K)

Solvent	Solubility (10^3 x M)
Benzene	2.36
Toluene	4.03
Hexane	0.072
Dichloromethane	0.36
Chloroform	0.71
Acetone	0.001
N,N-dimethylformamide	0.038
Tetrahydrofuran	0.08
Water	1.8×10^{-21}

Properties and reactivity of C_{60}

The chemical behavior of C_{60} is determined by its unique structural properties. First, C_{60} is not a super aromatic molecule, but actually the opposite. This is due to the fact that the molecule does not have delocalized double bonds, but instead it has alternating single and double bonds.[20] The double bonds are localized between two six-membered rings (6,6-bonds) and are exocyclic with respect to the five-membered rings. The bonds between five- and six-memebered rings are practically single bonds.[8] Second, there is a substantial angle strain (8.5 kcal/mol/per carbon atom) in the C_{60} because the angles deviate by 11.6 from the ideal value of 120 for sp^2-hybridized carbons.[21] Third, C_{60} has a very low reduction potential that can be explained by the fact that the molecule possesses three low-lying degenerate lowest unoccupied molecular orbitals (LUMOs).[22] It accepts up to six electrons reversibly.

The structural properties discussed above result in a general reactivity pattern that can be summarized in three main points:[20]

1. C_{60} reacts like an electron-deficient alkene
2. The relief of angle strain is the main driving force for addition reactions

3. Products with a double bond between a five and a six-memebered ring are avoided and this determines the regioselectivity of addition reactions.

Making C_{60} derivatives: Bingel-Hirsch reaction

It is possible to carry out a wide variety of reactions with C_{60}. Some examples are: Diels-Alder [4+2] cycloadditions[23] oxidative [3+2] cycloadditions,[24] [2+2] photochemical cycloadditions,[25,26] azide additions,[27] additions of azomethine ylides,[28] hydrogenations,[11] halogenations,[29] Michael additions,[30] and Bingel-Hirsch additions.[8, 17] Cyclopropanations have proven to be very efficient in the preparation of fullerene derivatives.[31] There are three main methods to produce methanofullerenes (cyclopropanated fullerenes): (1) thermal addition of diazo compounds followed by thermolysis or photolysis, (2) addition of free carbenes, and (3) reactions that proceed in by an addition-elimination mechanism.

An example of a reaction that proceeds by an addition-elimination mechanism is the Bingel-Hirsch reaction. This reaction is very useful due to the fact that it occurs under mild conditions and that it only produces methanofullerenes from addition across the double bond between two six-memebered rings in good yields (~40%).[8]

The classical conditions for this reaction are to add diethylbromomalonate and sodium hydride to C_{60}.[17] In the reaction, diethylbromomalonate is deprotonated by sodium hydride and the anionic nucleophile that is formed attacks C_{60}. The methanofullerene is obtained when Br^- is eliminated by cyclization. The mechanism is shown in Figure 3. Another way of carrying out this reaction is to produce the malonate in situ by treatment with carbon tetrabromide and base.[8]

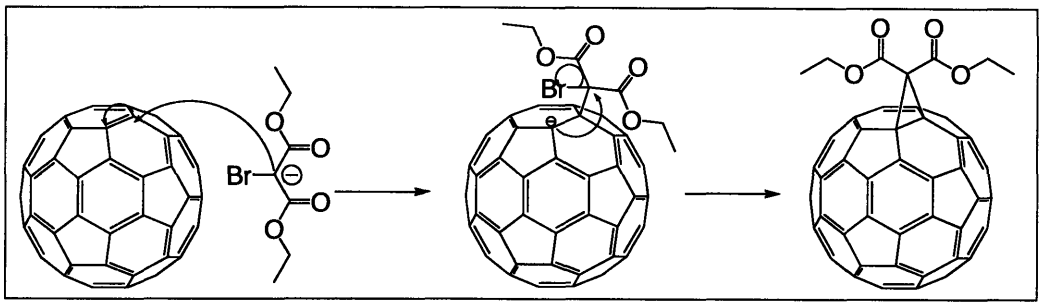

Figure 3. Bingel-Hirsch reaction mechanism.

This chapter describes the synthesis of various fullerene derivatives that can be used as fluorescence quenchers and that can also be used in various biosensors applications. All the C_{60} derivatizations were performed through the Bingel-Hirsch method because of the advantages previously discussed.

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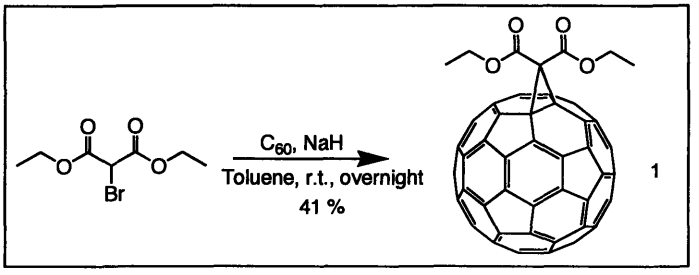
The goal of this project was the development of fullerene-based fluorescence quenchers for applications in various biosensing schemes that are currently being worked on in our group. These schemes are discussed in detail in the introduction to this thesis. In order to incorporate fullerene derivatives into biosensors, they need to posses a functional group that can be easily reacted with biomolecules. Two of the functional groups by which molecules are conjugated to biomolecules such as amino acids and proteins are amines and carboxylic acids. For this reason, our target was to synthesize amine- and carboxy-containing C_{60} that could then be conjugated to biomolecules.

Synthesis of a carboxy-containing C_{60}

The first compound we decided to synthesize was **1**. This selection was made based on the fact that this is the classical Bingel-Hirsch reaction, which has been very well studied.[1] This compound was also chosen because it would be a good starting point for further derivatizations. The reaction for the production of compound **1** is shown in Scheme 1. The details of this reaction are discussed in the introduction to this chapter.

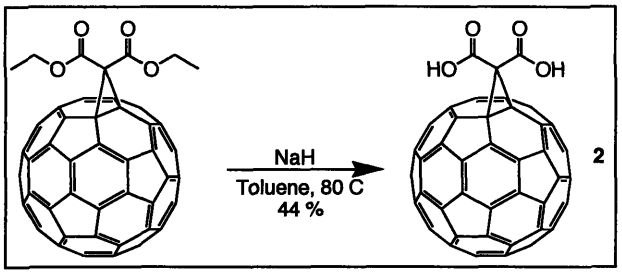
Scheme 1



From this compound, we made our first and only carboxylate-containing C_{60} (compound **2**). The reaction is shown in Scheme 2.[2] No more carboxylate-containing C_{60} derivatives were made because of two reasons. Firstly, the synthesis and the purification of **2** were very simple. The reaction proceeded smoothly under mild conditions and the

product was purified by precipitation with acid, followed by centrifugation. Secondly, compound **2** was useful for the intended application of conjugating it to an amine group of a biomolecule.

Scheme 2[2]



Synthesis of amine-containing C_{60}

Three amine-containing C_{60} derivatives (shown in Figure 1) were our main synthetic targets. These compounds were all chosen for different reasons. Compound **3** was chosen because its preparation had been published in literature.[3] This compound seemed very promising but we feared that the ester bonds might be cleaved under the reaction conditions needed for conjugation to biomolecules. Therefore, we decided to also prepare compounds **3** and **4**. These compounds have amide bonds, instead of ester bonds, which are more resistant to cleavage under the bioconjugation conditions. Furthermore, we decided to synthesize not only one compound with an amide bond instead of an ester bond, but two, one with a shorter chain (compound **3**) and another with a longer chain (compound **4**). The reason for this is that we thought that compound **4** would react more readily with the carboxylic group of a biomolecule because its amines are less sterically hindered.

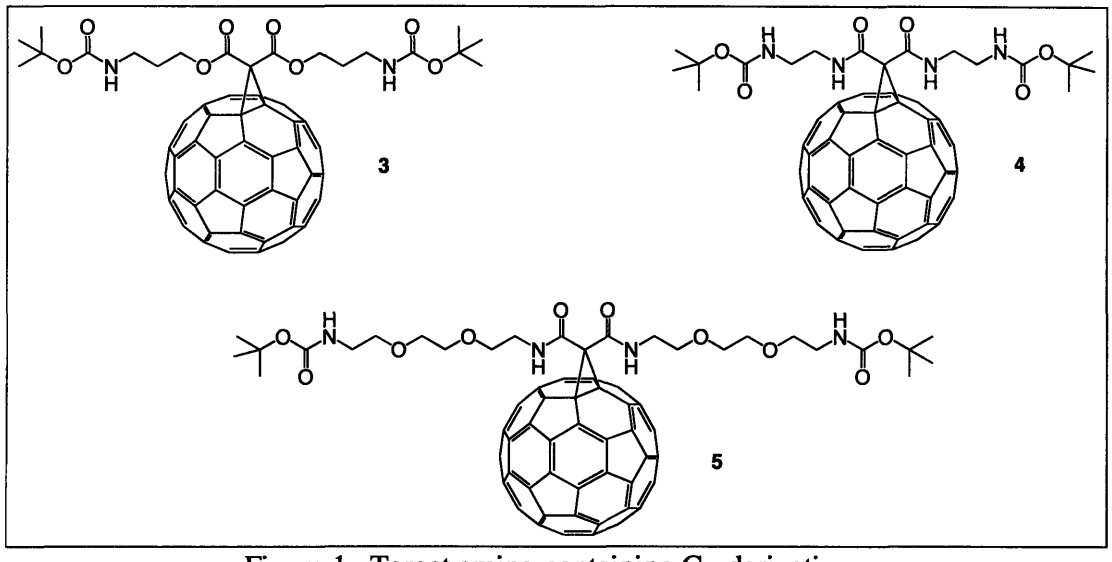
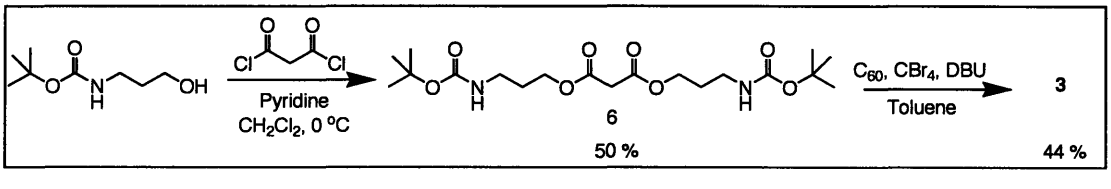


Figure 1. Target amine-containing C_{60} derivatives.

To make compound **3**, it was first necessary to synthesize malonate **6**. The starting materials were purchased and used as received. The reaction was carried out according to the conditions shown in Scheme 3. Compound **6** was purified by flash chromatography in silica gel with hexane: ethyl acetate 1:1 as the elution solvent. It was then reacted with C_{60} under Bingel-Hirsch conditions to produce compound **3**. The crude product was also purified by flash chromatography in silica gel to give a 44 % of the amine-containing methanofullerene, which is in the optimal range for Bingel-Hirsch additions (40-50%).

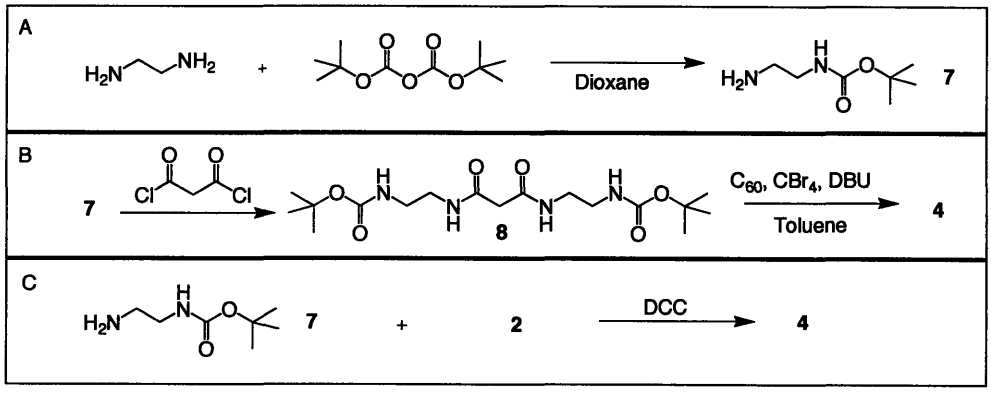
Scheme 3[3]



To make compound **4**, two different approaches were taken. The first reaction is the same in both cases (Scheme 4, A) and consists of the Boc-protection of diaminoethane to give compound **7**. This reaction was carried out according to a

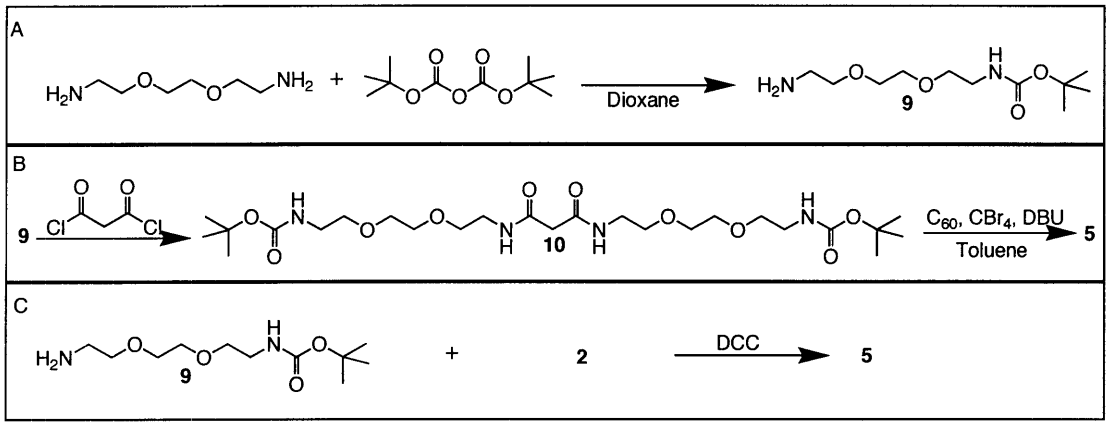
literature procedure.[4] The product was obtained in a 75 %. The first approach was a two-step reaction. First, compound **7** was reacted with malonyl dichloride to produce the malonate **8**, which would then be reacted with C_{60} under the Bingel-Hirsch addition conditions (Scheme 4, C). Various conditions were tried for this reaction and none produced malonate **8**. We then moved to the second approach. This was a one step reaction in which compound **7** was reacted with compound **2** in the presence of DCC (Scheme 4, C). The product obtained was insoluble in all the solvents tried. Unfortunately, due to this solubility problem, we were unable to characterize this compound.

Scheme 4



The analogous two approaches discussed above were used to produce compound **5**. As in the previous case, the first reaction of both approaches is the same (Scheme 5, A). This reaction is the mono-Boc protection of the amine to give compound **9** in 95% yield.[5] In the first approach, compound **9** was reacted with malonyl dichloride to produce the malonate **10**, which would then be reacted with C_{60} under the Bingel-Hirsch addition conditions to give compound **5**. Compound **9** was purified by flash chromatography on silica gel. In the second approach, compound **9** was reacted directly with compound **2** in the presence of DCC to produce compound **5**. Unfortunately, the product that resulted

from both methods was insoluble in all the solvents tried. Due to this solubility problem, this compound could not be characterized.



Synthesis of biotinylated-C_{60}

The biotin-streptavidin system has been applied to biosensor designs because of its large binding constant (K_d = 4 x 10^{-14} M)[6]. Given our interest of using fullerene derivatives for biosensor applications, we decided to synthesize biotinylated fullerenes. The synthesis of a biotinylated fullerene has been reported by Hirsch and coworkers.[7] Their synthesis was 7-steps long and yielded a mono-biotinylated fullerene. His synthetic scheme is shown in Scheme 6. We decided not to use this approach, because we could produce biotinylated fullerenes in just two or three steps from previously obtained products. Our target compounds are shown in Figure 2.

Scheme 6

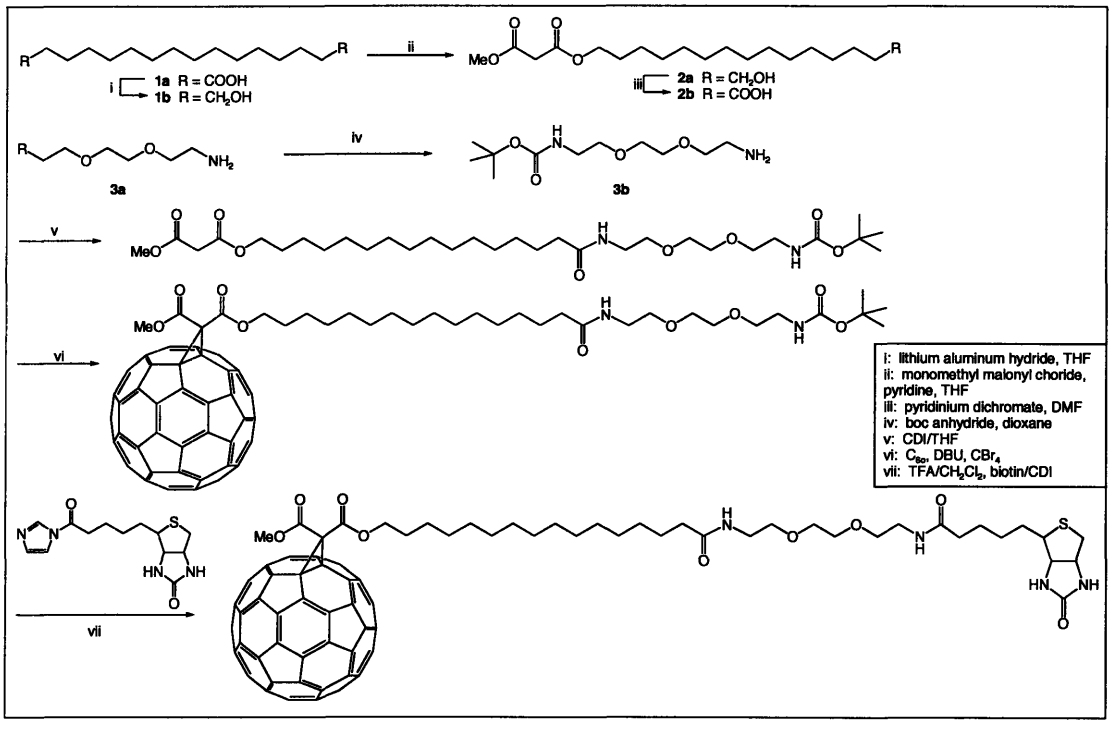
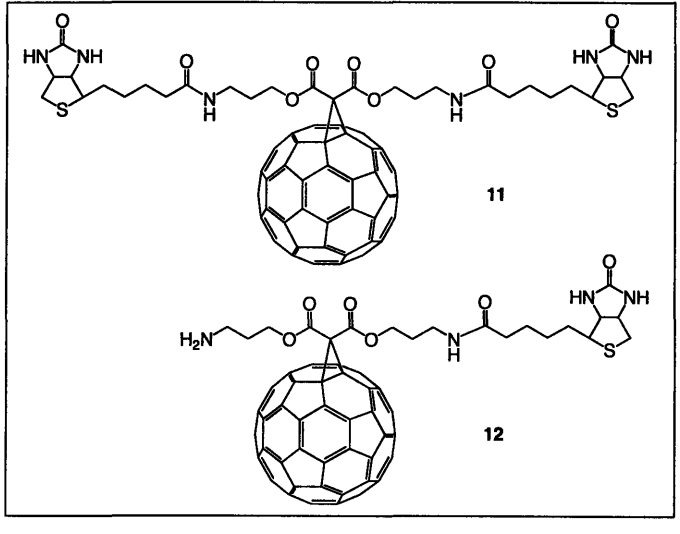


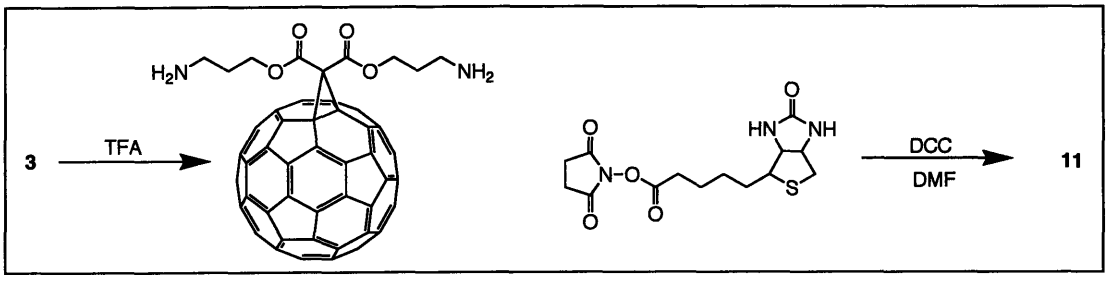
Figure 2. Target biotinylated-C_{60} derivative



To produce compound **11**, we started with compound **3**. First, we deprotected the amines and then reacted it with N-hydroxysuccinimide-biotin to obtain compound **11**. The reactions and conditions are shown in Scheme 7. Unfortunately, the product of this

reaction was insoluble in all the solvents tried. Due to this solubility issue, the compound could not be characterized.

Scheme 7



To produce compound **12**, we used the same approach as to produce compound **11**. In this case, we only used one equivalent of N-hydroxysuccinimide-biotin and of DCC. We thought that this compound would be more soluble than compound **11**, but unfortunately, the product obtained was insoluble. Due to this, compound **12** could not be characterized. Another possible explanation to why these two reactions failed might be that the DCC was not good. The DCC used was obtained from a very old bottle and it had formed a big pellet due to humidity. All the other starting materials were pure, so this is the only one we suspect could have been bad.

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[1] Bingel, C. *Chem. Ber.*, **1993,** 126, 1957-1959.
[2] Cheng, F.; Yang, X.; Zhu, H.; Sun, J.; Liu, Y. *J. Phys. Chem. Sol.* **2000,** 61, 1145-1148.
[3] Richardson, C.; Schuster, D.; Wilson, S.; *Organic Letters*, **2000,** 2(8) 1011-1014.
[4] Eisenführ, A.; Arora, P. S.; Sengle, G.; Takoka, L. R.; Nowick, J. S.; Famulok, M. *Bioorganic and Medicinal Chemistry* **2003,** 11, 235-249.
[5] Trester-Zedlitz, M.; Kamada, K.; Burley, S. K.; Fenyö, D.; Chait, B. T.; Muir, T. W. *J. Am. Chem. Soc.* **2003,** 125, 2416-2425.
[6] Green, N. M. *Methods Enzymol.* **1990,** *184*, 51-67.
[7] Braun, M.; Camps, X.; Vostrowsky, O.; Hirsch, A.; Endreβ, E.; Bayeryl, T. M.; Birkert, O.; Gauglitz, G. *Eur. J. Org. Chem.* **2000,** 1173-1181.

Experimental

Compound 1

This compound was prepared by following the general Bingel-Hirsch reaction conditions. Approximately 150mL of dry toluene were cannulated into a 250 mL round bottom flask under a nitrogen atmosphere. The flask was then charged with fullerene powder (1.50 g, 2.08 mmol), sodium hydride (0.78 g, 20 mmol) and diethylbromomalonate (0.35 mL, 2.2 mmol). The reaction was stirred for 7 h and then quenched with methanol. The crude was filtered and the toluene was rotovapped off. The crude brown solid was purified on silica gel (7:3 toluene/hexane) to provide the product as a brown solid (0.749 g, 0.854 mmol, 41%).[1] ^{1}H-NMR (300MHz, $CDCl_3$): δ = 4.57 (q, 4 H), 1.49 (t, 6 H). Found (ESI-MS) *m/z* = 878.0811. Calculated *m/z* = 878.1203.

Compound 2

Approximately 90mL of dry toluene were cannulated into a double neck 250 mL round bottom flask equipped with a condenser under nitrogen atmosphere. The flask was then charged with the diester fullerene (150 mg, 0.17 mmol) and NaH (0.90 g, 23 mmol). The reaction was stirred under nitrogen at 80°C for 10 h. The reaction was quenched by adding 3 mL of methanol dropwise, followed by the addition of 60 mL HCl. A brown precipitate was formed, which was filtered and washed in order with toluene, 2 M HCl, water and benzene. The brown solid was dissolved in methanol and centrifuged to remove insoluble impurities. The solvent was rotovapped off and the product (61 mg, 0.075 mmol, 44%) was dried under vacuum.[2] Found (ESI-MS) *m/z* = 822.5245. Calculated: 822.3037.

Compound 3

This compound was prepared by following the general procedure of the Bingel-Hirsch reaction. Approximately 100 mL of dry toluene were cannulated into a 250 mL round bottom flask under a nitrogen atmosphere. xThe flask was charged with the malonate above (87.3 mg, 0.217 mmol), fullerene powder (102 mg, 0.142 mmol), 1,8-Diazabicyclo[5_4_0]undec-7-ene (DBU, 62.3 _L, 0.417 _mol) and carbon tetrabromine (69.08 mg, 0.2083 mmol). The reaction was left stirring at room temperature for 1 h. It was quenched by adding 30 mL of amonioum chloride solution (10%). Purification of the product (71.0 mg, 0.0625 mmol, 44%) was achieved by running two flash chromatography columns (10:1 toluene/ethyl acetate and 1:1 toluene/ethyl acetate, respectively).[3] ^{1}H-NMR (300MHz, $CDCl_3$): δ 4.90 (bs), 4.57 (t), 3.32 (q), 2.06 (m), 1.46 (s). Found (ESI-MS) *m/z* = 1137.1797. Calculated *m/z* = 1137.1290.

Compound 6

Approximately 100 mL of dry dichloromethane were cannulated into a double neck 250 mL round bottom flask under a nitrogen atmosphere. The flask was charged with tert-butyl N-(3-hydroxypropyl) carbamate (0.97 mL, 5.7 mmol) and pyridine (0.45 mL, 5.6 mmol). The flask was left stirring in an ice bath until cold. Approximately 30 mL of dry dichloromethane were cannulated into a 50 mL round bottom flask. This flask was charged with malonyl dichloride (0.28 mL, 2.9 mmol). The contents of this flask were cannulated dropwise into the 250 mL round bottom flask. The reaction was left stirring overnight. The product (580 mg, 1.4 mmol, 50%) crude reaction was purified by column

chromatography on silica gel (1:1 hexane/ethyl acetate). [3] ^{1}H-NMR (300MHz, $CDCl_3$): δ 4.85 (s, 1 H), 4.19 (t, 4 H), 3.37 (s, 2 H), 3.17 (q, 4 H), 1.82 (m, 4 H), 1.41 (s, 18 H). Found *m/z* = 419.2385. Calculated (ESI-MS) *m/z* = 419.2388.

Compound 7

Ethylenediamine (14 mL, 209 mmol) was dissolved in approximately 30 mL of dioxane and added to a 100 mL round bottom flask equipped with an addition funnel. Boc-anhydride (3.0 g, 14 mmol) was dissolved in 25 mL of dioxane, added to the addition funnel, and added to the flask dropwise over a period of 3 h. The reaction was stirred at room temperature for 30 h. Dioxane was rotovapped off, followed by the addition of 50 mL of water. This was extracted three times with 100 mL of dichloromethane and back-washed with 5 mL of water and 5 mL brine. The organic layers were collected, dried with magnesium sulfate and the solvent rotovapped off to give the product as a yellow oil (1.7 g, 10 mmol, 75%).[4] ^{1}H-NMR (300MHz, $CDCl_3$): δ 5.24 (s, 1 H), 3.18 (t, 2 H), 2.82 (t, 2 H), 2.34 (d, 2 H), 1.38 (s, 9 H). Found (ESI-MS) *m/z* = 161.1292. Calculated *m/z* = 161.1285.

Compound 9

Tris(ethylene glycol)-1,8-diamine (14 mL, 96 mmol) was dissolved in approximately 30 mL of dioxane and added to a 100 mL round bottom flask equipped with an addition funnel. Boc-anhydride (3.0 g, 14 mmol) was dissolved in 25 mL of dioxane, added to the addition funnel, and added to the flask dropwise over a period of 5 h. The reaction was stirred at room temperature for 24 h. Dioxane was rotovapped off, followed by the addition of 50 mL of water. This was extracted three times with 100 mL of dichloromethane and back-washed twice with 5 mL of water. The organic layers were collected, dried with magnesium sulfate and the solvent rotovapped off to give the product as a yellow oil (3.2 g, 13 mmol, 95%).[5] ^{1}H-NMR (300MHz, CD_3OD): δ 3.6 (s, 4 H), 3.54 (t, 2 H), 3.53 (t, 2 H), 3.24 (t, 2 H), 2.8 (t, 2 H), 1.4 (s, 9H). Found (ESI-MS) *m/z* = 249.1800. Calculated *m/z* = 249.1809.

Compound 10

9 (3.52g, 14.2mmol) and 2mL of triethylamine were dissolved in approximately 50 mL of chloroform in a 100 mL round bottom flask equipped with an addition funnel. This was placed in an ice bath and left stirring until cold. Malonyl dichloride (0.70mL, 7.1mmol) was dissolved in approximately 20 mL of chloroform in an addition funnel and added slowly to the round bottom flask. The reaction mixture was left stirring overnight, letting the reaction reach room temperature. The crude product was purified by flash chromatography on silica gel using hexane:ethyl acetate (1:10). The second running fraction was the product (2.15g, 3.7mmol, 52%). ^{1}H-NMR (300MHz, CD_3OD): δ 3.6 (s, 4 H), 3.54 (t, 2 H), 3.53 (t, 2 H), 3.24 (t, 2 H), 2.8 (t, 2 H), 1.4 (s, 9H). Found (ESI-MS) *m/z* = 587.3315. Calculated *m/z* = 587.3309.

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[1] Bingel, C. *Chem. Ber.*, **1993,** 126, 1957-1959.

[2] Chen, F.; Yang, X.; Zhu, H.; Sun, J.; Liu, Y.; *Journal of Physics and Chemistry of Solids,* **2000,** 61, 1145-1148.

[3] Richardson, C.; Schuster, D.; Wilson, S.; *Organic Letters*, **2000,** 2(8) 1011-1014.
[4] Eisenführ, A.; Arora, P. S.; Sengle, G.; Takoka, L. R.; Nowick, J. S.; Famulok, M. *Bioorganic and Medicinal Chemistry* **2003,** 11, 235-249.

[5] Trester-Zedlitz, M.; Kamada, K.; Burley, S. K.; Fenyö, D.; Chait, B. T.; Muir, T. W. *J. Am. Chem. Soc.* **2003,** 125, 2416-2425.

There has been an incredible growth in the past 20 years in the use of fluorescence in the biological sciences. It has increased, so that now it is used in numerous applications such as DNA sequencing, environmental monitoring, genetic analisis, clinical chemistry, flow cytometry, cellular localization and cell identification and sorting. This chapter attempts to describe fluorescence quenching and how it can be used in biosensor applications.

Fluorescence: a quick overview

Luminescence is the emission of light from an electronically excited state of a substance. It is divided into phosphorescence and fluorescence, depending on the excited state from which the emission takes place. If the emission takes place from a singlet state, the phenomenon is called fluorescence and if it takes place from a triplet state, it is called phosphorescence. These processes are usually depicted in a Jablonski diagram, as shown in Figure 1.

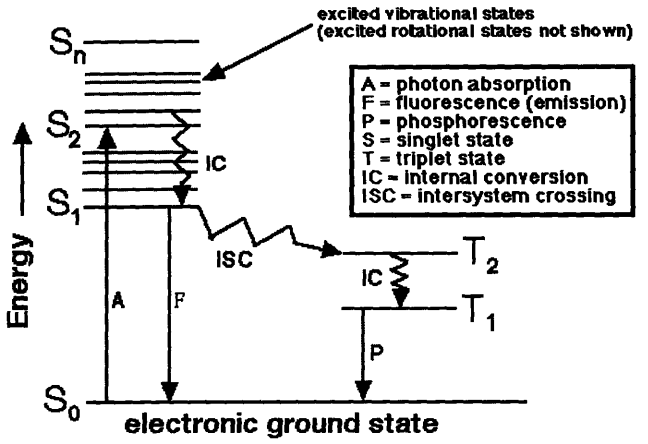

Figure 1. Jablonski diagram

The most relevant feature of fluorescence for this chapter is that it allows for high sensitivity detection.[1] This feature has been known for over 100 years. One example can be found in the use of the fluorescent marker fluorescein to demonstrate that the Danube and the Rhine rivers were connected in 1877.[2]

Fluorescence Quenching

Fluorescence quenching is the term used to define any process that decreases the fluorescence intensity of a sample. Quenching can be the result of many different interactions such as energy transfer, excited-state reactions, molecular rearrangements, collisional quenching and ground-state complex formation. The two types of quenching that are going to be discussed and seen throughout this chapter are dynamic and static quenching.

Dynamic quenching consists of a quencher diffusing to a fluorophore during the lifetime of its excited state. When contact between the two species occurs, the fluorophore returns to its ground state without emitting light. Static quenching consists of the formation of a nonfluorescent complex between the fluorophore and the quencher. For quenching to occur, there must be contact between the fluorophore and the quencher.

Fluorescence quenching has been very well studied and is used to provide information about biochemical systems. The requirement of contact between the fluorophore and the quencher is the key for all the biochemical applications. Quenching studies can be used to localize the quencher or the fluorophore in a cell and to determine the diffusion coefficient of the quencher.

Stern-Volmer Equation

The Stern-Volmer equation describes the dependence of fluorescence quenching on the quencher concentration and applies to both, static and dynamic quenching, at low quencher concentrations:

$$F_0/F = 1 + K_{SV}[Q]$$

F_0 is the fluorescence intensity when there is no quencher, F is the fluorescence intensity in the presence of quencher, [Q] is the concentration of quencher and K_{SV} is the Stern-Volmer constant.

The Stern-Volmer constant gives a quantitative measure of the quenching. K_{SV} has a different meaning in the case of dynamic and static quenching.[3] In dynamic quenching, K_{SV} is related to the fluorescence lifetime τ_0 and to the diffusion-controlled bimolecular rate constant k_d:

$$K_{SV} = k_d \tau_0$$

In static quenching, K_{SV} is the association constant for the complex:

$$K_{SV} = [FQ]/[F][Q]$$

Quenching data are usually shown as plots of F_0/F as a function of [Q]. This is known as the Stern-Volmer plot and it is a linear plot with a slope equal to K_{SV} and a y-intercept of 1. To distinguish between static and dynamic quenching, the dependence of K_{SV} on temperature can be measured. K_{SV} is expected to increase with temperature in the case of dynamic quenching because more collisions would take place and to decrease in the case of static quenching because of the dissociation of the complex. They can also be distinguished by measuring the lifetime. In the case of dynamic quenching, the lifetime

varies with varying concentrations of quencher and in dynamic quenching it stays the same.

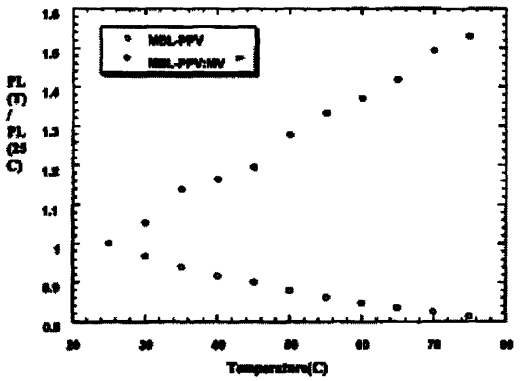

Figure 2. Comparison between static and dynamic quenching. The solid circles represent dynamic quenching, while the open circles represent static quenching.[3]

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[1] Lakowicz, J. R. *Principles of Fluorescence Spectroscopy* **1999,** Kluwer Academic / Plenum Publishers, New York.

[2] Berlman, I. B. *Handbook of Fluorescence Spectra of Aromatic Molecules*, 2nd ed, Academic Press, New York.

[3] Wang, J.; Wang, D.; Miller, E. K.; Moses, D.; Heeger, A. J. *Synthetic Metals* **2001,** 119, 591-592.

In the previous chapter, the synthesis of fullerene derivatives was described in detail. This is the first step towards the development of fullerene-based fluorescence quenchers. In this chapter, a description of the next steps needed to accomplish this goal is given. First, Stern-Volmer experiments were conducted to determine whether or not the fullerene derivatives would be good quenchers for our polymers. Second, a polymer with pendant fullerenes was made to determine whether or not there was an enhancement in the quenching as compared with the Stern-Volmer data. Third, the use of the biotin-streptavidin system to determine how well the fullerene derivatives would perform in a biosensor system is discussed.

Stern-Volmer Experiments

The polymers used for all the experiments described in this thesis were different derivatives of poly(phenylene-ethynylene) (PPE). These polymers produce signal amplification due to efficient excited state migration, which is facilitated by their semiconductive nature.[1]

The first Stern-Volmer experiments we carried out consisted of adding underivatized C_{60} to two toluene-soluble PPEs. This would give us an idea of the magnitude of the quenching constant between our polymers and the fullerene derivatives. The structure of the polymers used for these experiments is shown in Figure 1.

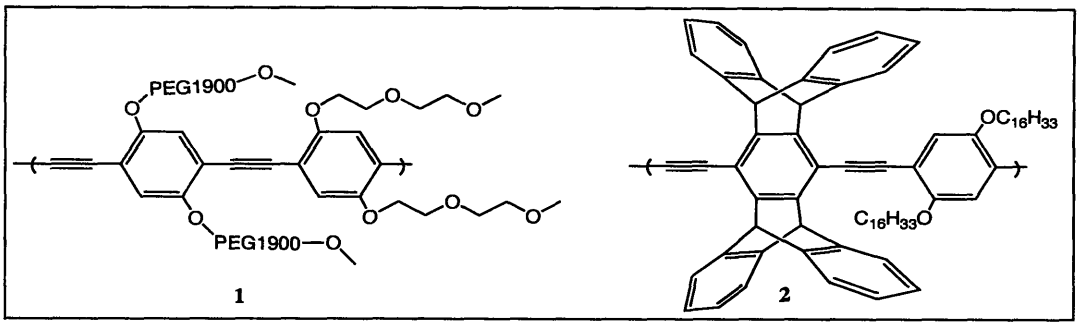


Figure 1. Structure of polymers **1** and **2**

There are no reports in literature of quenching studies between C_{60} or its derivatives and PPEs. There is, however, a report of solution quenching studies between poly(phenylene-vinylene) (PPV) derivatives and C_{60} derivatives.[2] The Stern-Volmer data and the polymer and quencher used in this article are shown in Figure 2. The reported K_{SV} for their system is 2.5 x 10^3 M^{-1}, which is very large and suggests a strong interaction between the polymer (MEH-PPV) and the quencher (TCM-C_{60}).

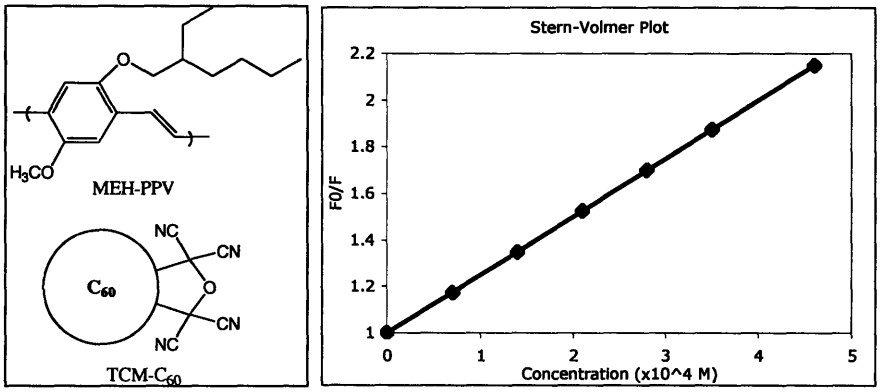


Figure 2. Polymer (MEH-PPV) and quencher (TCM-C_{60}) structures used by Zheng *et. al* (a) and Stern-Volmer data for their system (b).

Polymer **1** was chosen for these experiments because it is a normal alkyl-chain derivatized PPE that is soluble in toluene. Solubility in toluene was required due to the fact that C_{60} is almost exclusively soluble in this solvent. The quenching data and the Stern-Volmer plot for polymer are shown in Figure 3. The K_{SV} for this system is 6 x 10^6, which is three orders of magnitude higher than the K_{SV} reported by Zheng and coworkers.

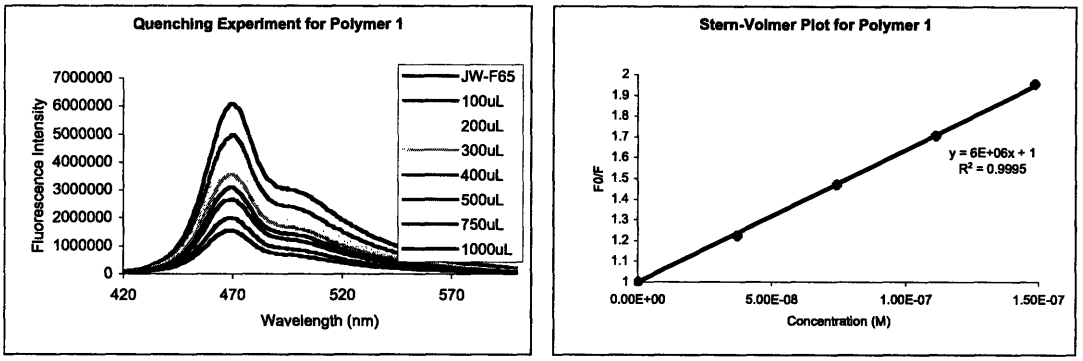


Figure 3. Quenching data and Stern-Volmer plot for polymer **1**

Polymer **2** was chosen for these experiments because it is soluble in toluene, but more importantly, because it is a pentiptycene-derived PPE. Besides providing more quenching data about the PPE-C_{60} system, we wanted to check if the quenching of this polymer was greater than that of polymer **1**. The rational behind this is that, based on the three-dimensional structure of the pentiptycene unit, it seems that it can serve as a "cradle" for the C_{60}. Therefore, we thought there could be some preferential quenching of this polymer as compared with the "normal" PPE. Unfortunately, the K_{SV} for this system (7 x 10^6 M^{-1}) was very similar to the K_{SV} for polymer **1** (6 x 10^6 M^{-1}), which suggests that no preferential quenching of this polymer is occurring.

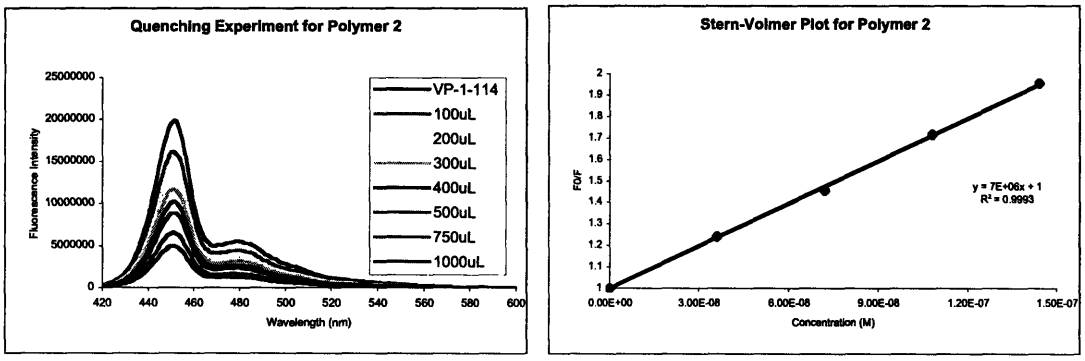


Figure 4. Quenching data and Stern-Volmer plot for polymer **2**

Given that such large quenching constants were found for these two PPE-C_{60} systems, we decided to do some experiments with PPEs and derivatized C_{60}. The

structures of the two polymers and the fullerene derivative used in these experiments are shown in Figure 5. The quenching data and the Stern-Volmer plots for these two systems are shown in Figures 6 and 7.

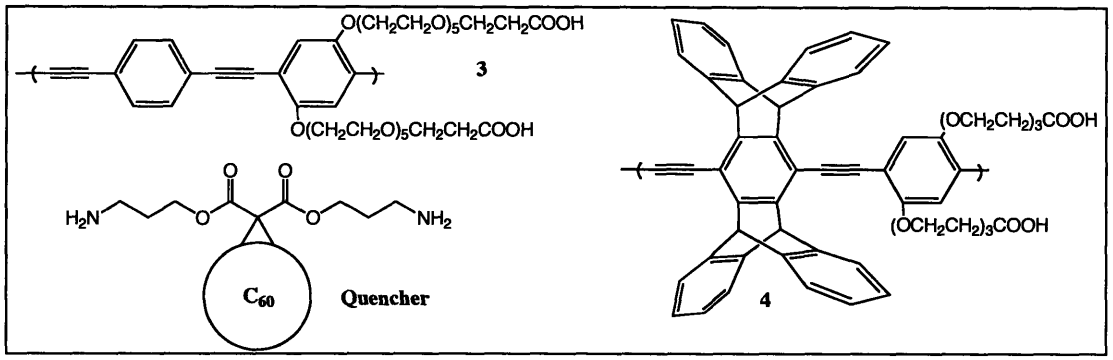


Figure 5. Structure of polymers **3** and **4** and the fullerene derivative used as the quencher.

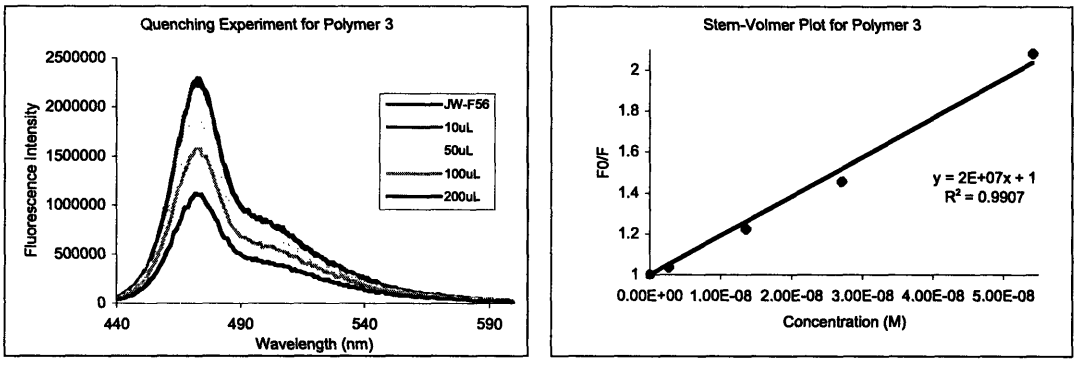


Figure 6. Quenching data and Stern-Volmer plot for polymer **3**.

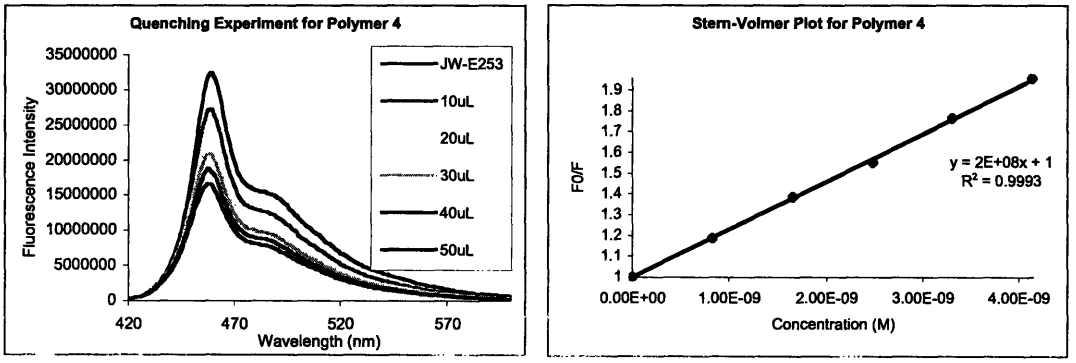


Figure 7. Quenching data and Stern-Volmer plot for polymer **4**.

The Stern-Volmer constants for the four systems described above are very large (>1 x 10^6 M^{-1}). The type of quenching happening in these experiments was static quenching. This can be derived from the following equation discussed in the introduction to this chapter: $K_{SV} = k_d \tau_0$. The lifetime of the four polymers used was approximately 0.5ns. The maximum value for the diffusion-controlled bimolecular rate constant is the bimolecular diffusion-controlled coefficient, which is 1 x 10^{10} $M^{-1}s^{-1}$. Therefore, if the quenching was solely dynamic, the maximum the K_{SV} value could be is 5 M^{-1} (K_{SV} = (1 x 10^{10} $M^{-1}s^{-1}$)(0.5 x 10^{-9} s). This proves that the major type of quenching happening is static quenching. Nevertheless, lifetime experiments were performed and the results were that the lifetime of the polymers remained constant (0.5ns) regardless of the concentration of quencher. As discussed in the introduction to this chapter, this is a definite proof that the type of quenching occurring is static.

The selection of the excitation wavelength played a key role in the quenching data obtained for polymers **1** and **2**. This was due to the fact that there was absorption from the C_{60} at the excitation and emission wavelengths, which means that there was competitive absorption. The relative absorptions of polymers **1-4** and of C_{60} are shown in Figure 8A. It seems that the absorption of C_{60} at the polymers excitation wavelengths is not significant, but it is at the concentration range used for the experiments. Only the data for polymer **2** will be shown, given that the data for polymer **1** is analogous and including it, would not contribute anything additional to this discussion. In 8B, the absorption spectra of polymer **2** are shown before and after adding the quencher (C_{60}). It is clear from this graph that the quencher absorption is, in fact, significant.

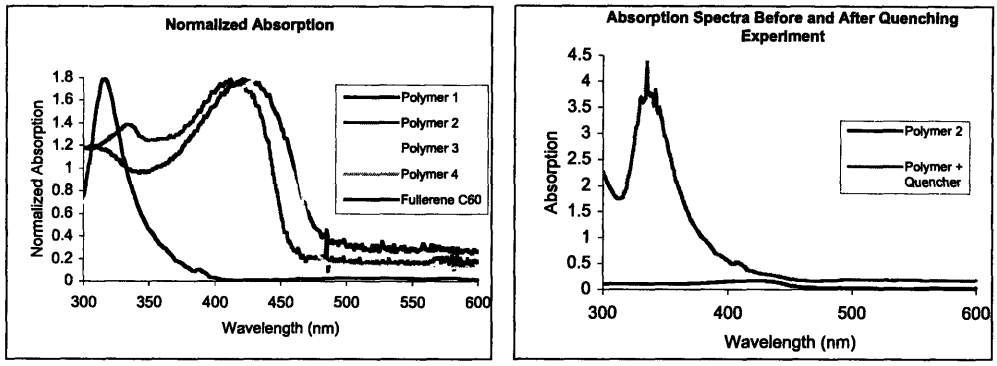


Figure 8. Normalized absorption for polymers 1-4 (A). Absorption spectra before and after quenching experiment for polymer 2 (B).

A correction for competitive absorption was used by Zheng et. al.[2] and the equation they used to calibrate the fluorescence intensity is shown below:

$$F = F_{em} \cdot \frac{1 - e^{\wedge}(-\varepsilon_1 C_1 l)}{\varepsilon_1 C_1} \cdot \frac{\varepsilon_1 C_1 + \varepsilon_2 C_2}{1 - e^{\wedge}(-(\varepsilon_1 C_1 + \varepsilon_2 C_2) l)} \cdot \frac{\varepsilon_3 C_3 l}{1 - e^{\wedge}(-\varepsilon_3 C_3)}$$

where F is the fluorescence intensity after calibration, F_{em} is the experimental fluorescence, C_1, ε_1 and C_2, ε_2 are the concentration and molar extinction coefficients of the polymer and C_{60} at the excitation wavelength, C_3 and ε_3 are the concentration and molar extinction coefficient of C_{60} at the emission wavelength. Their data is shown in Figure 9.

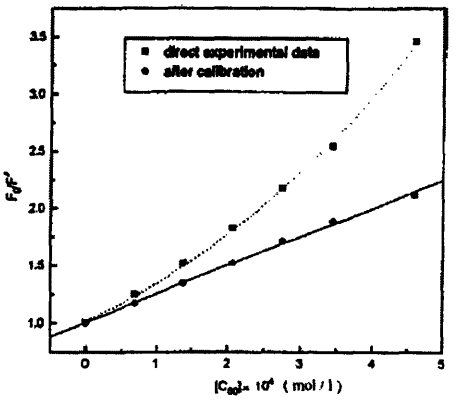


Figure 9. The dependence of F_0/F' on the concentration of C_{60}

The equation discussed above was used to correct the quenching data obtained for polymer **2**, when an excitation wavelength of 406nm was used. The absorption maximum for polymer **2** is 413nm, but 406nm was chosen because it allowed for starting the fluorescence scan at a shorter wavelength. The corrected and uncorrected Stern-Volmer plots are shown in Figure 10A. The Stern-Volmer plot shown in Figure 4 for polymer **2** was obtained after processing data collected when exciting the polymer at 425nm. At this wavelength, the absorption by C_{60} is very small. This Stern-Volmer plot is shown again in Figure 10B, to make the comparison between the two easier. The results using the calibration equation and using a different excitation wavelength are the same. In both cases, the slope is $7 \times 10^6\ M^{-1}$, but a better correlation coefficient is obtained when exciting the polymer at 425nm. Given these results, we decided to excite the polymer at 425nm and not perform the correction for competition absorption.

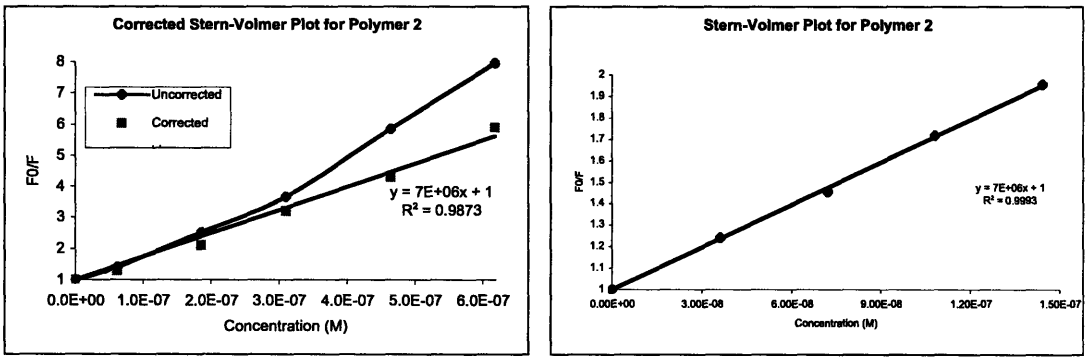


Figure 10. Corrected and uncorrected Stern-Volmer Plots for Polymer **1**.

The competitive absorption between the polymer and the quencher (C_{60}) can be explained as follows. When the quencher was added to the polymer, there was a decrease in the fluorescence intensity, but not all of it could be attributed to quenching. The fact that there is a significant absorption from the quencher at the polymer excitation wavelength would result in a "filter" effect because fewer photons would be available to

excite the polymer. This idea is depicted in Figure 11. In 11A, there are no quencher molecules present and all the photons are available to excite the polymer. The absorption of these photons results in an emission from the polymer. In 11B, there are quencher molecules present, which absorb part of the photons. These photons are not available to excite the polymer, thus resulting in a smaller absorption by the polymer and subsequently in a smaller emission from it. In 11C, the same result as in 11B is shown, but in this case a filter is used instead of the quencher molecules.

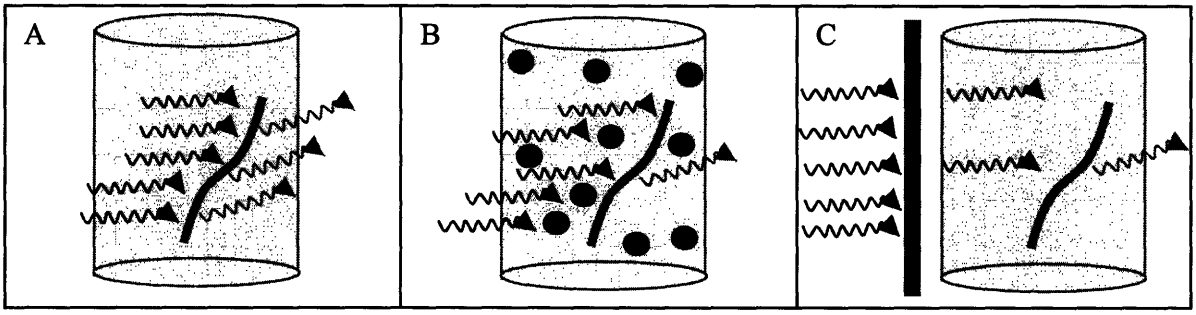


Figure 11. Filter effect by quencher molecules. (A) No quencher molecules are present and all the photons are available for polymer excitation. (B) Quencher molecules are present and absorb part of the photons, thus making these inaccessible to the polymer. (C) A filter is used to show the effect that the quencher molecules produce.

Making fullerene-pendant polymer

In the previous section, we confirmed that C_{60} and its derivatives are very good fluorescence quenchers for PPEs. Since the goal of this project was to make highly quenching fullerene derivatives for biosensor applications, the next logical step to take is to make a fullerene-pendant polymer. In the introduction to this thesis, various biosensor applications were described. One of them, the turn-on of fluorescence by quencher removal (Figure 12) shows why making this kind of polymer is the next step towards the achievement of our goal. In this type of biosensor, the quencher is covalently attached to the polymer by a linker. Depending upon the nature of this linker, whether it is labile

under certain conditions or reacts with a certain chemical, it can be used as a functional biosensor.

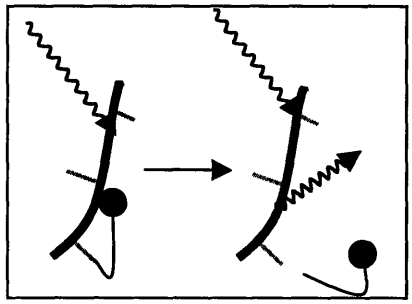
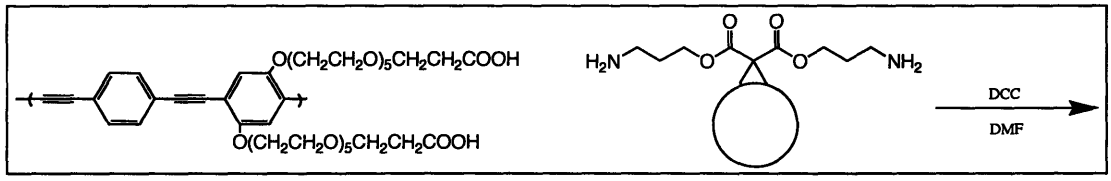


Figure 12. Turn-on of fluorescence by quencher removal.

The goal of this particular set of experiments was to determine whether the quenching was larger when the fullerene molecules were attached to the polymer or if it was the same as when they were free in solution. The coupling reaction performed was that shown in Scheme 1. It was followed by making a time-based fluorescence acquisition. The general procedure for all these experiments was to add 3mL of polymer solution into a cuvette, activate the carboxylic groups in the polymer for about an hour, add the amine-derivatized C_{60} and then monitor the reaction.



Scheme 1. DCC coupling reaction

The kinetic profiles for four of the experiments performed are shown in Figure 13. The conditions for each experiment are shown in Table 1. In experiment A, polymer **3** was activated with an excess of DCC, followed by the addition of one equivalent of quencher. Note that each polymer repeat unit has two carboxyl groups and each molecule of quencher has two amine groups. In experiment B, polymer **3** was also activated with an excess DCC, followed by the addition of one equivalent of quencher on

four different occasions. In experiment C, polymer **3** was activated with four equivalents of DCC, followed by the addition of four equivalents of quencher. In experiment D, polymer **3** was activated with 20 equivalents of DCC, followed by the addition of four equivalents of quencher.

Table 1. Reaction conditions.

Experiment	Compounds	Equivalents	Time of addition of 11 (s)
A	Polymer 3	1	400s
	DCC	20	
	Quencher	1	
B	Polymer 3	1	120s, 1920s, 2785s, 3095s
	DCC	2	
	Quencher	1*	
C	Polymer 3	1	120s
	DCC	4	
	Quencher	4	
D	Polymer 3	1	300s
	DCC	20	
	Quencher	4	

*See text for conditions

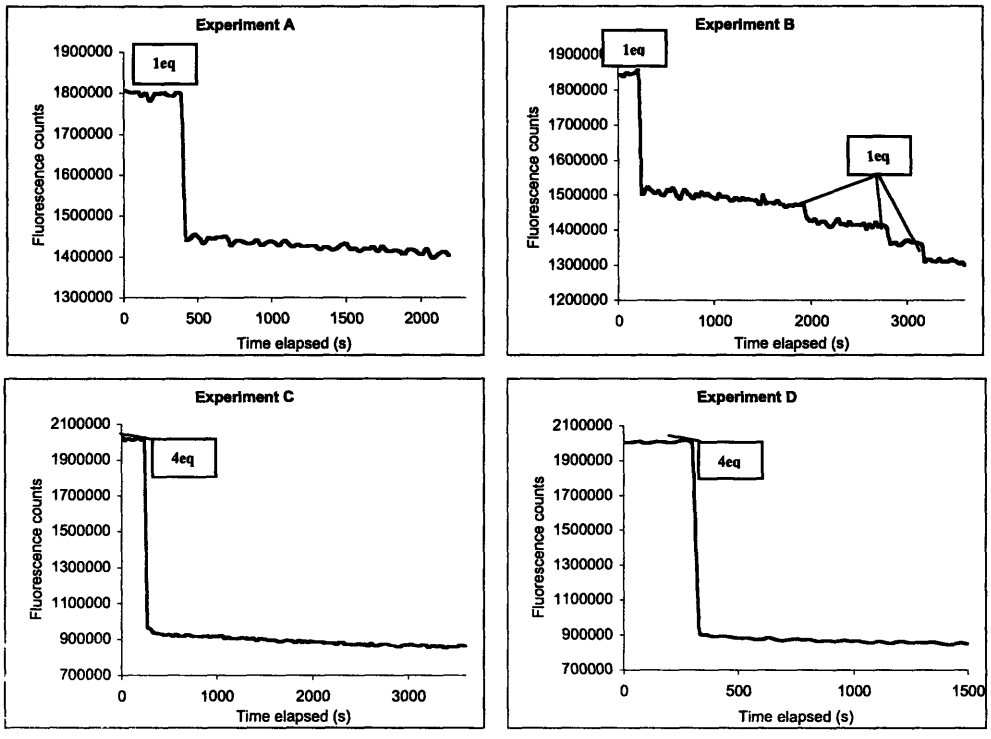


Figure 13. Time-based fluorescence acquisition monitoring coupling reaction. Conditions for each experiment are given in Table 1.

There are two main conclusions that can be drawn from the kinetic profiles of the coupling reactions. The first one is that the reaction occurs very rapidly. This conclusion can be made from the fact that the fluorescence intensity drops sharply when the fullerene molecule is added and continues to drop slowly as time goes by in all of the experiments. The second conclusion is that a large excess DCC (about 4 equivalents) is needed for the coupling reaction to go to completion. In experiments B-D, four equivalents of the fullerene derivative where added and the difference between the experiments was the amount of DCC added. The decrease in fluorescence was lower in experiment B, where 2 equivalents of DCC were added and the same for experiments C and D, where 4 and 20 equivalents were added, respectively.

We expected the fluorescence quenching to be larger when the fullerene molecules were covalently attached to the polymer than when they were free in solution. Our reasoning was that by covalently attaching the fullerene molecules to the polymer, we were increasing the local concentration of quencher molecules around the polymer. We say the local concentration increases because the total concentration is unchanged. This idea is depicted in Figure 14. In 14A, the fullerene molecules are free in solution because no DCC has been added. In 14B, DCC has been added and the coupling reaction has started. This causes more fullerene molecules to be in close proximity to the polymer, thereby increasing the concentration of fullerene molecules close to it. In 14C, the DCC coupling reaction has come to completion and the concentration of quencher molecules around the polymer is at its maximum.

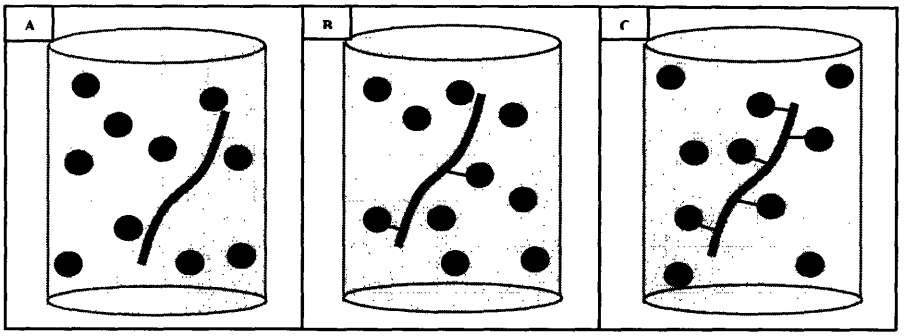


Figure 14. Increasing the local concentration of quencher molecules. (A) No DCC has been added and the fullerene molecules are free in solution. (B) DCC has been added and the coupling reaction has started. (C) The coupling reaction is complete.

To determine whether the quenching was larger when the fullerene molecules were covalently attached to the polymer, we measured the fluorescence of two samples. They both contained the same concentration of polymer and fullerene and the difference between them was that DCC was added to one of the samples and not to the other. The sample to which DCC was added is called "Attached molecules" in Figure 15 and the one to which DCC was not added is called "Free molecules". As we can see from Figure 15, there is a greater decrease in the fluorescence intensity of the "Attached molecules". In fact, there is only a 6% decrease in the fluorescence intensity when the fullerene is added to the polymer and a 30% decrease in the fluorescence intensity when DCC is added under the same conditions.

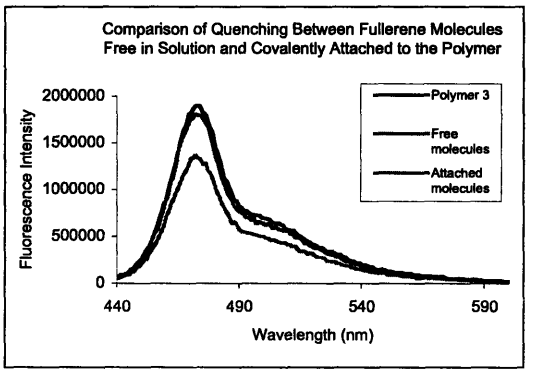


Figure 15. Comparison of quenching between fullerene molecules free in solution and fullerene molecules covalently attached to the polymer.

Control experiments were performed for the DCC activation and for dilution. The fluorescence intensity was not significantly affected while and after adding DCC to the polymer solution (Figure 16A). Also, the intensity was not significantly affected by the dilution that occurred when the fullerene solution was added to the polymer solution. To check for this, we added the same volume that was added of fullerene solution for the coupling reaction of pure solvent (Figure 16B).

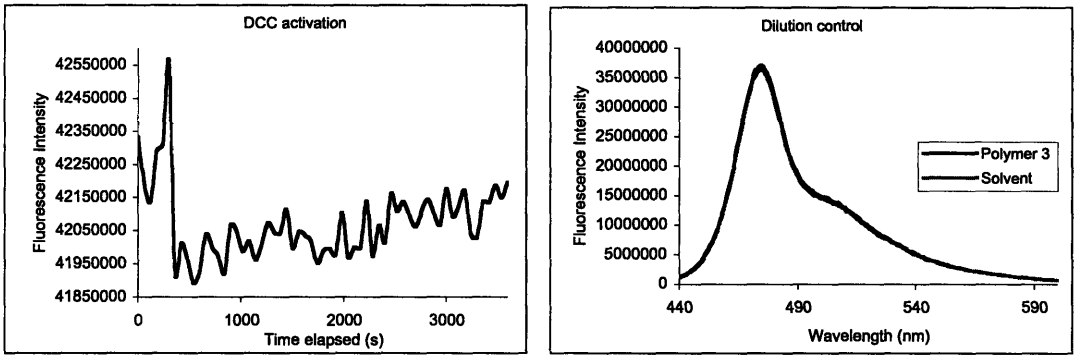


Figure 16. Control experiments. (A) Time-based acquisition during DCC activation. (B) Dilution control experiment

Calixarene experiments

Calixarenes are cyclic oligomers of p-t-butylphenol and formaldehyde (Figure 17). They are known to complex C_{60}. We decided to carry out an experiment to determine if we could turn back on the fluorescence of the polymer with pendant fullerenes by adding calixarene.

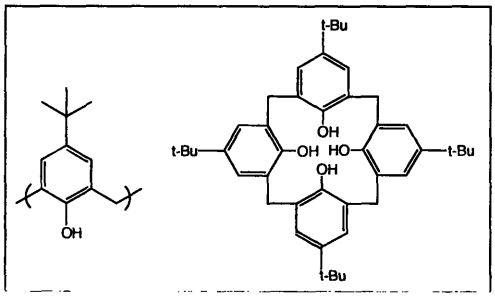


Figure 17. General calixarene structure (left) and calyx[4]arene.

There are two ways to represent the C_{60} molecules when they are attached to the polymer. One of them is for the polymer and the C_{60} to be in close proximity to each other (Figure 18, middle) and the other is for them to be further apart (Figure 18, left). For quenching to occur, the quencher molecules must be in close proximity to the polymer. Therefore, the best representation of the covalently attached C_{60} molecules is that in which polymer and C_{60} are close together. We thought the formation of the C_{60}-calixarene complex would pull the C_{60} away from the polymer and that this would turn the polymer fluorescence back on (Figure 18, right).

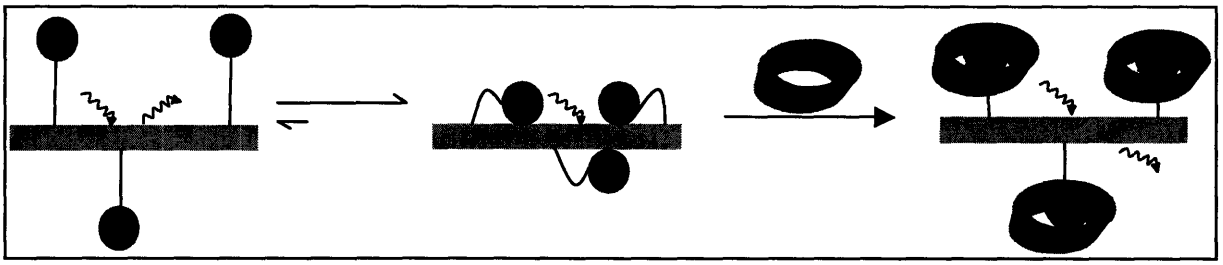


Figure 18. Calixarene experiments. On the left, the fullerene molecules are represented apart from the polymer. In the middle, the fullerene molecules are in close proximity to the polymer and this allows for quenching to occur. On the right, calixarene is added and the C_{60} molecules are pulled away from the polymer, which turns the polymer fluorescence on.

Different aliquots of calixarene solution were added to a solution of polymer with pendant fullerenes and the fluorescence intensity was measured after each addition. Unfortunately, the results were not the expected ones and the polymer fluorescence was not turned back on. The results are shown in Figure 19.

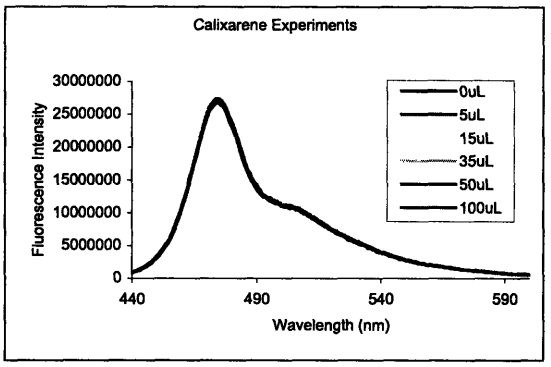


Figure 19. Fluorescence intensity when adding different amounts of calixarene solution.

Future Work: Biotin-Streptavidin Experiments

The biotin-streptavidin system has been applied to biosensor designs because of its large binding constant ($K_d = 4 \times 10^{-14}$ M) and it is used to determine whether or not a new element will work in a biosensor.[3] Because of this, the next step in the integration of a fullerene fluorescence quencher in a biosensor, should be to try it with the biotin-streptavidin system.

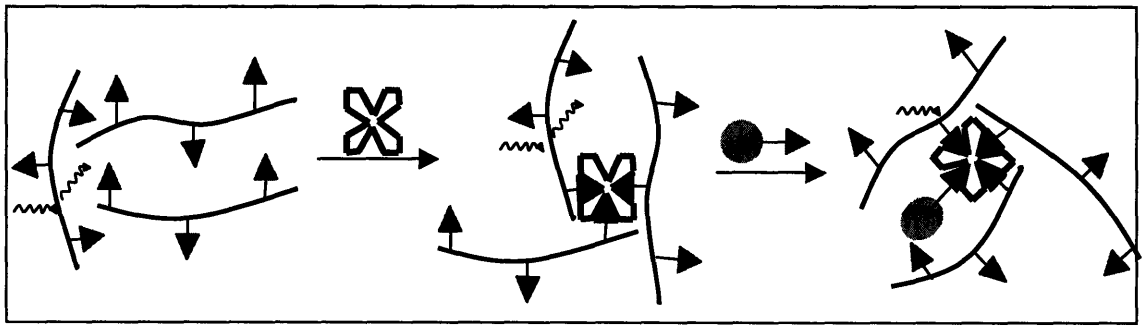


Figure 20. Biotin-Streptavidin experiments. Streptavidin is added to a biotinylated polymer and the fluorescence remains unchanged. On the left, biotinylated C_{60} is added to the solution and the fluorescence is quenched.

A simple experiment that can be performed is the following. A fluorescent biotinylated polymer would be added to a cuvette and its fluorescence would be measured. Then, streptavidin would be added to the polymer solution, so that it could bind the biotin units in the polymer and the fluorescence would be measured. No

significant changes in the fluorescence intensity should occur at this point. After this, biotinylated C_{60} would be added to the cuvette and the fluorescence would be measured again. A large diminution in the fluorescence intensity is expected. This is due to the fact that streptavidin has four biotin binding sites and it would bring the polymer and the C_{60} in close proximity to each other. This idea is depicted in detail in Figure 20.

---

[1] Moon, J. H.; Swager, T. M. *Macromolecules* **(2002)** 35, 6086-6089.
[2] Zheng, M.; Bai, F.; Li, F.; Li, Y.; Zhu, D. *Journal of Applied Polymer Science* **(1998)** 70, 599-603
[3] Green, N. M. *Methods Enzymol.* **1990**, *184*, 51-67.

Experimental

General. Fluorescence spectra were measured with a SPEX Fluorolog-2 fluorometer (model FL112, 450W xenon lamp). The spectra in solution were obtained at room temperature using a quartz cuvette with a 1cm path length.

Materials. All solvents were spectral grade unless otherwise noted. C_{60} and biotin were purchased from Alfa Aesar and used as received. Streptavidin was purchased from Molecular Probes Inc. and used as received. All other chemicals were purchased from Aldrich Chemical In. and used as received.

General Protocol for Stern-Volmer Experiments. Polymer solutions with absorptions of 0.1 or less were prepared and 3mL were added to a quartz cuvette with a 1cm path length. Aliquots of quencher solution were added to this and the fluorescence was measured after each addition.

Polymer 1

Solutions:

Polymer 1 Solution: 1.001 mg of polymer 1 were dissolved in 50 mL of toluene

Quencher Solution: 4.440 mg of C_{60} were dissolved in 4 mL of polymer 1 solution

Instrument Parameters:

Parameter	Value
Scan Start	435nm
Scan End	650nm
Increment	1.0nm
Excitation	425nm
Integration Time	0.1s
Signals	Sc/Rc
Excitation Slit	1.103nm
Emission Slit	1.208nm
HV	950V

Procedure:

3 mL of polymer solution were added to a quartz cuvette and the fluorescence spectrum of the solution was taken. Aliquots of 100, 200, 300, 500, 750 and 1000 μL of quencher solution were added to the cuvette and the fluorescence was measured after each addition.

Polymer 2

Solutions:

Polymer 2 Solution: 0.368 mg of polymer 2 were dissolved in 50 mL of toluene

Quencher Solution: 3.562 mg of C_{60} were dissolved in 4 mL of polymer 2 solution

Instrument Parameters

Parameter	Value
Scan Start	435nm
Scan End	650nm
Increment	1.0nm
Excitation	425nm
Integration Time	0.1s
Signals	Sc/Rc
Excitation Slit	0.945nm
Emission Slit	0.998nm
HV	950V

Procedure:
3 mL of polymer solution were added to a quartz cuvette and the fluorescence spectrum of the solution was taken. Aliquots of 100, 200, 300, 500, 750 and 1000 μL of quencher solution were added to the cuvette and the fluorescence was measured after each addition.

Polymer 3
Solutions:
Polymer 3 Solution: 0.280 mg of polymer 3 were dissolved in 50 mL DMF
Quencher Solution: 0.233 mg of Quencher (structure shown in Fig. 5, Chapter 2) were dissolved in 3 mL of polymer 3 solution

Instrument Parameters

Parameter	Value
Scan Start	435nm
Scan End	650nm
Increment	1.0nm
Excitation	425nm
Integration Time	0.1s
Signals	Sc/Rc
Excitation Slit	0.998nm
Emission Slit	1.208nm
HV	950V

Procedure:
3 mL of polymer solution were added to a quartz cuvette and the fluorescence spectrum of the solution was taken. Aliquots of 10, 50, 100 and 200 μL of quencher solution were added to the cuvette and the fluorescence was measured after each addition.

Polymer 4
Solutions:
Polymer 4 Solution: 0.5 mL of stock solution of polymer 4 (0.44 mM in repeat units) were diluted with 50 mL DMF.
Quencher Solution: 0.146 mg of Quencher (structure shown in Fig. 5, Chapter 2) were dissolved in 2.5 mL of polymer 4 solution.

Instrument Parameters

Parameter	Value
Scan Start	435nm
Scan End	650nm
Increment	1.0nm
Excitation	425nm
Integration Time	0.1s
Signals	Sc/Rc
Excitation Slit	1.208nm
Emission Slit	1.208nm
HV	950V

Procedure:
3 mL of polymer solution were added to a quartz cuvette and the fluorescence spectrum of the solution was taken. Aliquots of 10, 20, 30, 40 and 50 μL of quencher solution were added to the cuvette and the fluorescence was measured after each addition.

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Education

Massachusetts Institute of Technology — Cambridge, MA
Candidate for Science Masters in Chemistry, June 2004.
Coursework: Principles of Bioinorganic Chemistry, Biological Chemistry II, Chemistry of Biomolecules, Advanced Biological Chemistry, Molecular Structure and Reactivity, Biophysical Chemistry, Chemical Tools for Assessing Biological Function.
Thesis: Developing highly quenching fullerene derivatives for biosensor applications. **GPA: 4.3/5.0**

University of Puerto Rico — San Juan, PR
Senior Thesis: Pharmacokinetic study of the plasma concentration of Nelfinavir (Viracept) in plasma of HIV and hepatitis C co-infected patients. **GPA: 3.75/4.0**

Experience

January 2003 - Present
Department of Chemistry, MIT — Cambridge, MA
Advisor: Timothy Swager
Research Assistant. Synthesized and characterized various fullerene derivatives for biosensor applications such as detection of DNA and proteins. Used these fullerene derivatives as super fluorescence quenchers of conjugated polymers.

February 2003 - May 2003
Department of Chemistry, MIT — Cambridge, MA
Teaching Assistant. Taught Organic Chemistry to freshmen and sophomores. Conducted recitations twice a week to clarify, explain and stimulate students. Graded problem sets and exams.

August 2001- May 2002
University of Puerto Rico – Medical School — San Juan, PR
Research Assistant. Developed an HPLC/UV method for the determination of Nelfinavir in human plasma. Performed pharmacokinetic studies in HIV and hepatitis C co-infected patients and determine the interactions of Nelfinavir and Rebetron®.

Summer 2001
Abbott Laboratories — North Chicago, IL
Summer Intern. Developed a method for particle size determination based in laser-light scattering.

June 2000 - May 2001
University of Puerto Rico — San Juan, PR
Research Assistant. Developed a suitable method for the determination of organic contaminants in rainwater. The compounds were extracted using Solid Phase Micro-Extraction (SPME) and characterized by GC/MS.

Skills
Experience with GC/MS, HPLC/MS, HPLC/UV, FT-IR, NMR, MALDI-TOF, fluorimeters, general laboratory equipment, synthesis of organic compounds and polymers, handling radioactive material and wet chemistry. Bilingual English/Spanish.

Awards, Honors
American Chemical Society (2000-2004); MIT Presidential Fellowship (2003); United States Achievement Academy (USAA) National Award for Outstanding Academic Performance (2002), Minority Access for Research Career Fellowship (2001), Golden Key National Honor Society (2001); Alliance for Minority Participation Fellowship (2000).

Citizenship
US Citizen

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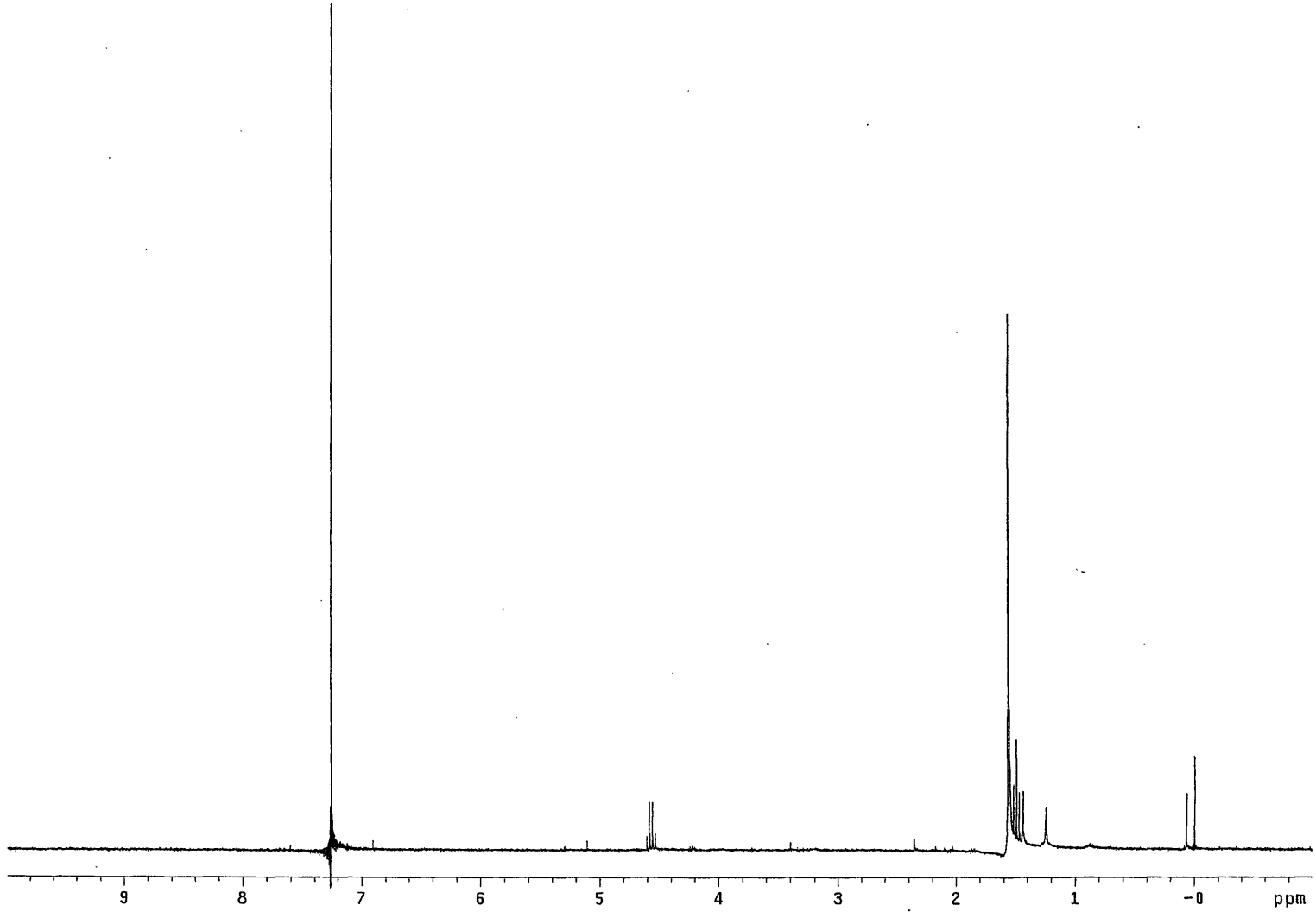
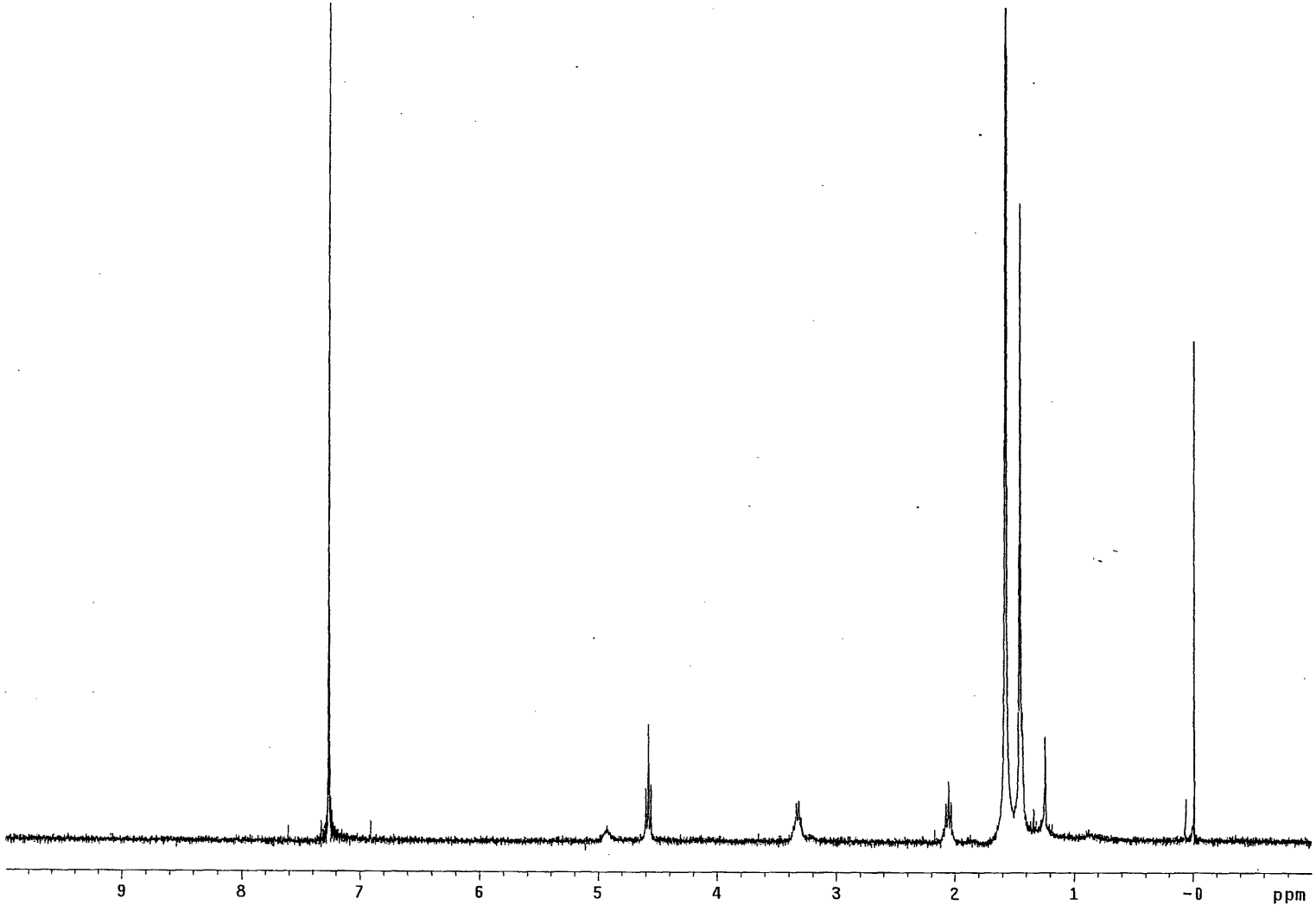
Appendix: NMR Spectra

Compound 1



Compound 3



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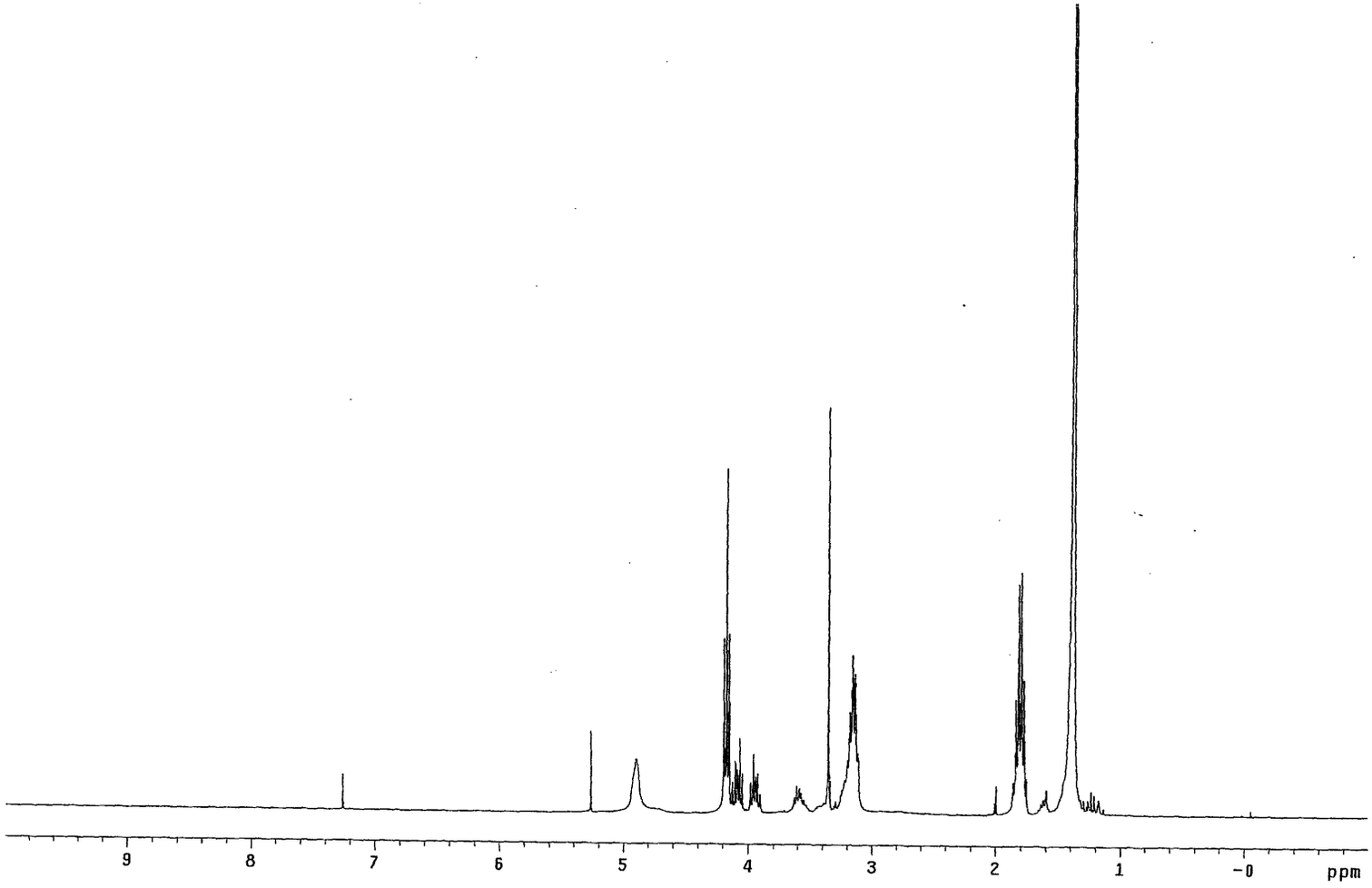

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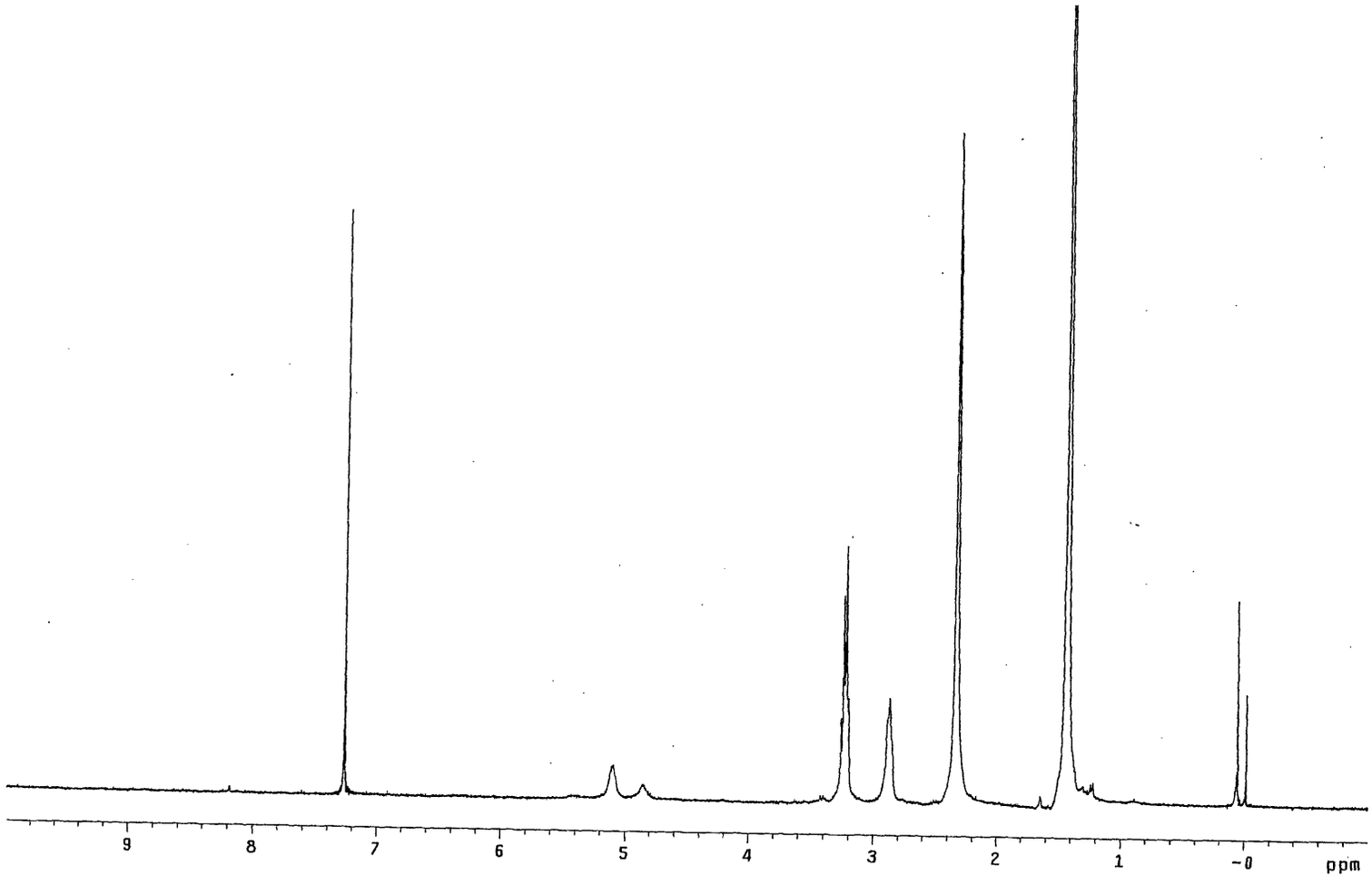
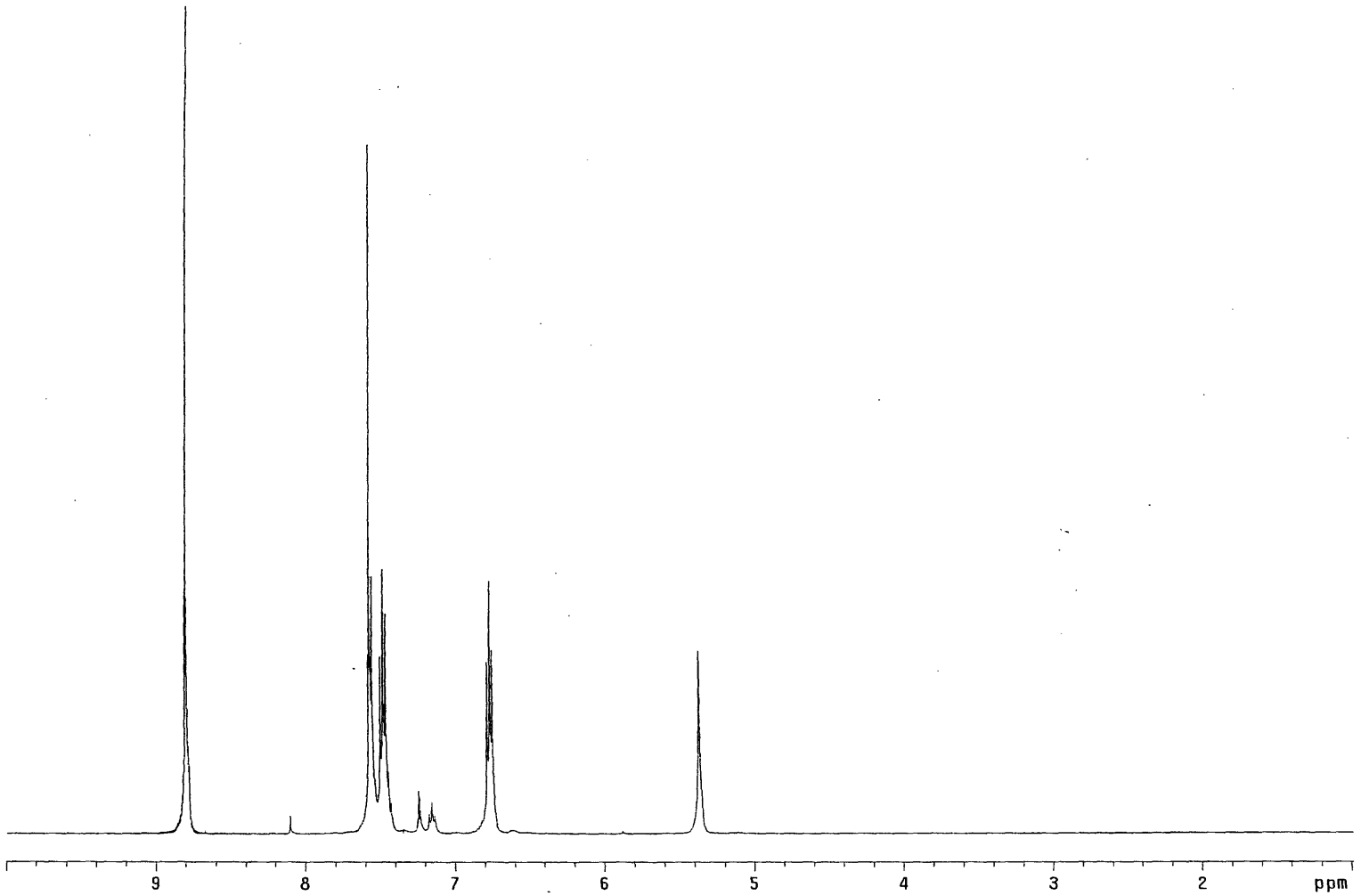


Compound 9



Compound 10